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The Actual Nitrogen Sources for Growth of Heterotrophic Bacteria in Non-limiting Media

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There is a large amount of information available about the minimal nutritional needs of different bacterial species and about the ability of certain bacteria to use one compound in place of another for growth; however, very little information is available about the compounds that are actually utilized for growth when the bacteria are growing in a complex medium offering alternatives for the essential nutrients.

Animals are fairly stringently restricted in their ability to utilize various nitrogenous compounds for growth. Nevertheless, when rats were fed on ^{15}N-labelled ammonia together with their normal diet, the ^{15}N was found in many of the amino acids of their tissue proteins, including some of the amino acids known to be essential in the diet; no ^{15}N was, however, found in lysine (Foster, Schoenheimer & Rittenberg, 1939; Rittenberg, Schoenheimer & Keston, 1939; Sprinson & Rittenberg, 1949). Different species of bacteria have widely differing nitrogenous needs, and it was thought of interest to determine what was the actual nitrogen source for growth of heterotrophic bacteria, either able or unable to use ammonia as sole nitrogen source, when growing in a medium containing peptides, free amino acids and ammonia. The only directly relevant evidence in the literature appears to be that *Escherichia coli* was found to utilize preformed amino acids in preference to synthesizing them from either carbon dioxide (Abelson, Bolton & Aldous, 1952) or glucose (Bolton & Abelson, 1952; Abelson, 1954), and that *Streptococcus faecalis* R utilized lysine peptides in addition to free lysine when growing in a partial protein hydrolysate (Pader, Melnick & Oser, 1948).

In this work, two strains of bacteria of widely differing nutritional needs were studied. In Expts. I and II the uptake of ammonia N from a complex medium was examined; in Expts. II and III the uptake of free lysine from the same medium was shown to be accompanied by an uptake of combined lysine; and in Expt. IV the effect of adding a single amino acid, lysine, to a medium containing ammonia as sole nitrogen source, was examined.

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METHODS

Selection of bacterial species. Two species of bacteria were needed, one capable of growing well with ammonia as sole source of nitrogen but not inhibited in any way in a rich medium, the other capable of growing only in a medium supplying practically all the amino acids as such. For the latter organism, *Leuconostoc mesenteroides* strain P. 60 was chosen, as Dunn, Shankman, Camien, Frankl & Rockland (1944) had shown that it needed some 18 amino acids for anything other than very scanty growth; a strain freshly obtained from the National Collection of Industrial Bacteria was used. The selection of an organism capable of good growth both on ammonia and on a rich medium proved more difficult, and, after several unsuccessful trials had been made with various stock cultures, a suitable organism was isolated from the rumen of a sheep by enrichment culture in, successively, an ammonium–glucose–lactate–salt broth and a peptone–yeast extract–meat extract–glucose–salt broth. The organism that grew best in both these media proved to be a hitherto undescribed coliform organism, which on routine bacteriological testing had the following properties: Gram-negative rod, usually about 1·8 μ. × 0·4 μ., though the length varied from 0·9 to 7·0 μ.; motile. Fermentations: in 24 hr., arabinose, dulcitol, galactose, glucose, maltose, mannitol, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose were fermented, with the production of acid and gas. After 3 days, acid and gas were formed from lactose and a slight acid reaction was obtained with aesculin and glycerol. No fermentation of dextrin, glycogen, inulin, inositol, raffinose or starch was found after 7 days. Growth was obtained in Koser's citrate medium. Nitrates were reduced to nitrites. Litmus milk showed no change in 24 hr., but acid, clotting and reduction of litmus occurred after 5 days. The indole test was negative on the third day; the Voges–Proskauer test was positive; the methyl-red test weakly positive. Gelatin was not liquefied. Urease was not formed. H_2S was produced after 48 hr.

A subculture of this organism has been deposited with the National Collection of Type Cultures (Colindale Avenue, London, N.W. 9) where it has been given the number 9711; it is referred to by that number below.

Culture media. Medium *A* contained Oxoid bacteriological peptone, 50 g.; Oxoid Lab-Lemco, 10 g.; Oxoid yeast extract, 10 g.; glucose, 50 g.; KH_2PO_4, 25 g.; sodium acetate (hydrated), 50 g.; salt solution ($MgSO_4,7H_2O$, 10 g.; $MnSO_4,4H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·5 g.; HCl, 3 drops; water to 250 ml.), 25 ml.; tap water to 5 l., pH was adjusted to 7·0 and the whole autoclaved at 15 lb./in.^{2} for 20 min. Medium *B* contained L-lysine monohydrochloride, 0·5 g.; KH_2PO_4, 50 g.; Na_2HPO_4, 50 g.; salt solution (as above), 50 ml.; $(NH_4)_2SO_4$, 10·4 g.; tap water to 9 l., pH

was adjusted to 7·0 and the whole autoclaved at 15 lb./in.^{2} for 20 min. and mixed with 1 l. of a 20% (w/v) glucose solution separately sterilized by filtration through a porcelain candle.

Isotopes. ^{15}N was obtained as $^{15}NH_4NO_3$ through the Isotope Division, Ministry of Supply Atomic Energy Research Establishment, Harwell. It was converted into $(^{15}NH_4)_2SO_4$ for use. Uniformly labelled [^{14}C]L-lysine was obtained from the Radiochemical Centre, Amersham.

Analyses. Ammonia was estimated according to Conway & O'Malley (1942), the NH_3 being liberated with K_2CO_3 and absorbed in the boric acid indicator solution. Total nitrogen was estimated by the micro-Kjeldahl procedure of Chibnall, Rees & Williams (1943), the NH_3 being distilled off in the apparatus of Markham (1942) and trapped in the boric acid indicator solution of Conway & O'Malley (1942). Carboxyl nitrogen was estimated by the technique of Van Slyke, Dillon, MacFadyen & Hamilton (1941), with the citrate buffer, pH 2·5, and the apparatus of Synge (1951).

Lysine was estimated by the manometric method of Gale (Gale & Epps, 1944; Gale, 1945), with a modified lysine-decarboxylase preparation (private communication from Dr G. Ellinger). In this, the medium employed for the growth of *Bacterium cadaveris* contained 50 g. of Oxoid Lab-Lemco, 50 g. of Oxoid bacteriological peptone, and 25 g. of NaCl in 5 l. of tap water; it was filtered after steaming for 30 min. at pH 8·4, the pH was adjusted to 7·8–8·0, 7·5 g. of glucose was added and the whole autoclaved at 15 lb./in.^{2} for 20 min. After growth for 24 hr. at 26° the bacteria were separated in a Sharples Supercentrifuge, washed, made into a thick suspension and poured into 8 vol. of ice-cold acetone. After preliminary centrifuging, the deposit was washed on a Büchner funnel with acetone, acetone–ether and ether, dried at room temp. and stored in the refrigerator for 3–4 days while arginase activity disappeared. For use, the Warburg flasks held 1·0 ml. of solution containing 0·5–1·0 mg. of L-lysine at pH 6·0, 1·5 ml. of 0·2 M sodium phosphate buffer (pH 6·0), and 0·5 ml. of a suspension containing 20 mg. of acetone-dried powder/ml. of buffer; when the reaction was complete, combined CO_2 was liberated with 0·25 ml. of 8 N-H_2SO_4 and the results were calculated with reference to a standard curve prepared from control experiments with known amounts of L-lysine carried out at the same time.

Radioactivity was estimated by counting samples at infinite thickness with an end-window Geiger–Müller counter and 1 cm.^{2} planchets, at least 2000 counts being made. ^{15}N was estimated by first converting the material into $(NH_4)_2SO_4$ and then into N_2, the ^{15}N abundance being determined in the mass spectrometer at the Rowett Research Institute by Dr E. I. McDougall or in the A.R.C. mass spectrometer at Reading University by Dr D. H. Tomlin.

Preparation of samples for analysis. Cultures were centrifuged in a Sharples supercentrifuge, and the deposits washed three times with water at 2°, in an M.S.E. refrigerated centrifuge, at 4500 rev./min. for 90 min. The deposits were then left to stand overnight in 5% (w/v) trichloroacetic acid (TCA) at 2°. They were then washed once with more cold TCA and treated three times with 200 ml. of 5% TCA at 90° for 10 min., followed by cooling and centrifuging, and finally washed with cold 5% (v/v) acetic acid, then with cold 1% acetic acid, after the technique of Gale & Folkes (1953). The extracted cells were hydrolysed with 500 ml. of 6 N-HCl under reflux for 24 hr., and the HCl was removed by repeated evaporation *in vacuo*. The hydrolysate was then filtered and a portion removed for preparation of amide and ninhydrin nitrogen, as described below.

The remaining hydrolysate was then treated with prepared charcoal, 3 g./100 ml. of hydrolysate, according to Partridge's (1949) method. The adsorbed tyrosine and phenylalanine were eluted from the charcoal with 5% (w/v) phenol in 20% (v/v) acetic acid, and the solution, after extracting the phenol with ether, was evaporated to dryness. After extracting the phenylalanine with cold water, the tyrosine was recrystallized from hot water, and nitrogen samples were prepared after measuring the radioactivity.

The cleared hydrolysate was then put on to the ion-exchange resin Zeo-Karb 215 (Permutit Co. Ltd., London), column sizes VI and VII of Partridge & Brimley (1952); the amino acids were displaced with 0·15 N-NH_4OH. As the amount of material used nearly saturated the columns, the first amino acids came off quite soon. Four or five large fractions were collected before the NH_3 front left the columns, a small fraction of about 10–15 ml. just as the NH_3 front passed out, and a further 250 ml. This last fraction was found to contain lysine free of other basic amino acids and nearly free of neutral amino acids. The fractions were tested on one-dimensional paper chromatograms, with either phenol–ammonia–water or collidine–water as solvents, after Synge (1951). The first and second fractions usually contained virtually all the glutamic and aspartic acids with some contamination of neutral amino acids: the last fraction before the NH_3 front contained neutral amino acids, mainly valine, the leucines, proline, alanine and glycine, with little contamination of either acidic or basic amino acids. These three main fractions, lysine, aspartic and glutamic acids and neutral amino acids were further purified by ionophoretic fractionation in the four-compartment cell described by Synge (1951). Short exposure times (2–3 hr.) were used for the basic and acidic amino acids, and long exposure times (6–8 hr.) for the neutral amino acids, after which the contents of the cathode compartment, the acetic acid compartment, and the specimen compartment respectively were collected. Where paper chromatograms still showed impurities, this procedure was repeated. In the last two experiments reported here, the lysine was further purified by making the picrate, recrystallizing, and regenerating lysine as the hydrochloride after Rice (1949). After measuring the radioactivity of these three fractions, nitrogen samples were prepared.

Ammonia, derived mainly from amide groups, was recovered from the original filtered hydrolysate by making alkaline with 0·5 ml. of 40% (w/v) NaOH and steam-distilling into a slight excess of H_2SO_4. The residue after this distillation was concentrated *in vacuo*, adjusted to about pH 3 with H_2SO_4 and made up to 5 ml.: 2 ml. of this solution was put in a Thunberg tube with 100 mg. of ninhydrin and 100 mg. of citrate buffer (pH 2·5) of Van Slyke *et al.* (1941). After evacuation, the tube was placed for 14 min. in a boiling-water bath, cooled and gassed three times with H_2S, shaking well each time (MacFadyen, 1944). The contents were then filtered and the precipitate was washed with buffer solution prepared by dissolving 1 g. of the above citrate buffer in 20 ml. of water. The combined filtrate and washings were made alkaline and steam-distilled into a slight excess of H_2SO_4. About 65% of the non-

amide nitrogen of the filtered hydrolysate was recovered as $(NH_4)_2SO_4$ in this way, and is referred to below as ninhydrin nitrogen; it is presumed to be representative of the amino acid nitrogen.

RESULTS

Uptake of ammonia nitrogen from rich medium A

In two experiments, Expts. I and II, $(^{15}NH_4)_2SO_4$ was added to medium *A* before sterilization at a concentration indicated in Table 1. Each 5 l. flask of medium was then inoculated with about 7–8 % of its volume of an 18 hr. culture of either organism 9711 or *Ln. mesenteroides*. In Expt. I, the two flasks were then incubated for 24 hr. without agitation, so that conditions would be relatively anaerobic. In Expt. II, a slow current of $O_2 + CO_2$ (95:5, v/v) (British Oxygen Co. Ltd.) was bubbled through the medium and then absorbed in saturated baryta solution; a solution of [^{14}C]lysine (10 μc in 0·25 mg.) was added in six portions at intervals over the 24 hr. incubation period. Analyses at the end of the incubation period are given in Table 1, and show that both organisms, though 9711 more than *Ln. mesenteroides*, produced ammonia from the culture medium. Mass spectrometer readings are given in Table 2; these show that about 6–7 % of the total nitrogen of the cells (Expt. II) and about 4 % of the nitrogen of the cell protein (Expt. I) of both organisms was derived from ammonia, to a large extent confirming with respect to the nitrogen moiety the finding of Abelson and co-workers with respect to the carbon moiety that bacteria do not synthesize amino acids when these are supplied preformed in the medium. The pattern of ^{15}N distribution in the various amino acids was essentially similar in both bacteria to that found by Foster *et al.* (1939) in the tissues of rats fed [^{15}N]-ammonia. As had been expected, *Ln. mesenteroides* took up less ^{15}N into its amino acids than did 9711; however, it took up considerably more into amide groups. No ^{15}N was incorporated in the lysine of either organism.

Uptake of free lysine from medium A

The results of the radioactivity measurements in Expt. II are given in Table 3. There was no evidence for anything more than minimal metabolism of the lysine. The activity of the lysine of the medium,

Table 1. *Analysis of medium A and cells after incubation*

Each 5 l. flask of medium contained initially 8600 mg. of total N, 485 mg. of total NH_3 N and 83 mg. of excess ^{15}N as NH_3 (Expt. I), or 8600 mg. of total N, 540 mg. of total NH_3 N and 76 mg. of excess ^{15}N as NH_3 (Expt. II); in Expt. II the total lysine content of the medium, measured after hydrolysis with 6 N-HCl for 24 hr. in a sealed tube at 105°, was 3100 mg. (590 mg. of N). The results shown are the total amounts found from the respective 5 l. flask.

	Expt. I		Expt. II	
	Organism 9711	*Ln. mesenteroides*	Organism 9711	*Ln. mesenteroides*
Ammonia N content of medium (mg.)	620	540	670	600
Total N content of washed cells (mg.)	—	—	213	200
Total N content of TCA-extracted cells (mg.)	152	155	153	143

Table 2. *Uptake of* NH_3 N *from medium A*

Incorporation of ^{15}N into the medium, cells and fractions after incubation of the two bacterial strains in medium *A* containing $(^{15}NH_4)_2SO_4$ is expressed as atom % excess ^{15}N. The normal ^{15}N abundance, measured with the Rowett Research Institute mass spectrometer, was 0·36 %; the figures have an error (95 % confidence limits) of about 5 % of the reading, except for the figures for tyrosine N and aspartic and glutamic acid N in Expt. II, where the error was probably 15–20 %; the figures for lysine N in Expt. II were determined with the mass spectrometer at Reading University, where the normal ^{15}N abundance was measured as 0·365 % with a 95 % confidence error of about 1 %.

	Incorporation of ^{15}N			
	Expt. I		Expt. II	
	Organism 9711	*Ln. mesenteroides*	Organism 9711	*Ln. mesenteroides*
Ammonia N of medium	10·8	10·7	9·0	9·8
N of washed bacterial cells	—	—	0·58	0·69
N of TCA-extracted cells	0·46	0·45	—	—
Amide N	—	—	3·4	5·8
Ninhydrin N	—	—	0·14	0·02
Lysine N	0·01	0·02	0·00	0·00
Tyrosine N	0·12	0·04	0·4	0·1
Aspartic + glutamic acid N	0·54	0·04	0·5	0·1
Neutral amino acid N	—	—	0·05	0·05

Table 3. *Metabolism and uptake of* [^{14}C]*lysine*

Radioactivity of medium, evolved CO_2 and amino acid fractions from bacterial cells, after incubation of bacteria in medium *A* containing [^{14}C]lysine (Expt. II) is given as counts/min. at infinite thickness, less background.

	Radioactivity (counts/min.)	
	Organism 9711	*Ln. mesenteroides*
Cell-free medium	143	133
$BaCO_3$	0·7	0·8
Neutral amino acids	6·8	3·2
Aspartic + glutamic acid	3·8	0·8
Lysine	1908	1621
Lysine of medium (calc.)	7700	7300

calculated from the known total lysine concentration (Table 1) and the total activity of the culture, was considerably higher than that of the lysine isolated from the cells, suggesting that unlabelled, and hence presumably peptide, lysine had been utilized as well as free lysine; synthesis of lysine from unlabelled precursors seems unlikely since no ammonia nitrogen was incorporated. Since, however, the method of calculation of the activity of the lysine of the medium was subject to considerable error, and since there was some evidence for the presence of a small amount of a non-nitrogenous impurity in the isolated cell lysine, this experiment was repeated with organism 9711 only. In Expt. III, medium *A* was used, without added [^{15}N]-ammonia and with a small (0·1 %) inoculum, but otherwise as in Expt. II. At the conclusion of the experiment, 500 ml. of cell-free medium was hydrolysed with 6 N-HCl for 18 hr. under reflux, evaporated to dryness and extracted with 0·5 % (v/v) HCl in absolute ethanol (Baliga, Krishnamurthy, Ragagopolan & Giri, 1955). The extract was then evaporated to dryness, taken up in 200 ml. of water, filtered and put on to a Zeo-Karb column; lysine was isolated in the usual way. In this experiment, and in Expt. IV mentioned below, the lysine was further purified through the picrate as already described. The radioactivity of the lysine of the cells was 2600 counts/min.; the radioactivity of the lysine of the medium was 1150 counts/min. However, the ratio of free lysine to total lysine (measured after hydrolysis with 6 N-HCl in a sealed tube for 24 hr. at 105°) was 1:4 at the conclusion of the experiment. It proved impossible to measure with any accuracy the initial small amount of free lysine, but the ratio of free to total amino acids, measured as the ratio of carboxyl nitrogen before and after hydrolysis as above, was 1:27 at the start of the experiment, compared with 1:5 at the conclusion, and there seems no reason to suspect that lysine would behave markedly differently from the other amino acids in this respect. Hence at all times throughout the incubation free lysine in the medium constituted less, probably considerably less, than one-quarter of the total lysine, so that the radioactivity of the free lysine would have been at least four times that of the lysine measured, probably more than twice that of the lysine isolated from the cells. Hence in the synthesis of protein, at least one peptide lysine molecule was used for every free lysine molecule. In view of the similarity of the results for the two organisms shown in Table 3, it is probable that a similar ratio would hold for *Ln. mesenteroides*.

Uptake of ammonia and of lysine from synthetic medium B

In Expts. I–III ammonia nitrogen constituted about 6 % of the total nitrogen available for growth; the effect of growing organism 9711 in a medium in which ammonia nitrogen constituted 96 % of the total nitrogen, the remainder being supplied as lysine, was investigated in Expt. IV. A volume (10 l.) of medium *B* was prepared, with $(^{15}NH_4)_2SO_4$ recovered from Expts. I and II. The inoculum was 1 ml. of a 24 hr. culture in a similar, but unlabelled, medium and incubation was continued for 40 hr. with $O_2 + CO_2$ bubbling through the medium. About 17 % of the total nitrogen of the medium was incorporated into the bacteria, about three-quarters of this remaining after TCA extraction, hydrolysis and filtration. By necessity, the largest part of the cell protein must have been synthesized from ammonia. Table 4 shows the ^{15}N abundance in the medium and the amino acid fractions of the cells after growth, and the calculated proportion of each

Table 4. *Uptake of ammonia* N *from medium B by organism* 9711

A very small inoculum of organism 9711 was grown in 10 l. of medium *B* containing initially 2200 mg. of total ammonia N (labelled with ^{15}N) and, by calculation, 77 mg. of lysine N. After growth, the washed bacterial cells contained 380 mg. of N, of which 280 mg. remained after TCA extraction, hydrolysis and filtration (Expt. IV). The readings were made with the mass spectrometer at Reading University, and have a 95 % confidence error of about 1 %, except for the figures for amide N and ninhydrin N, which were determined with the Rowett Research Institute mass spectrometer, which had an error of about 5 %. All the readings were made after incubation.

	^{15}N content (atom % excess)	% N derived from NH_3 of medium
Ammonia of medium	8·97	(100)
Amide N	7·84	88
Ninhydrin N	8·24	92
Neutral amino acid N	8·67	97
Tyrosine N	8·64	96
Aspartic + glutamic acid N	7·20	81
Lysine N	0·164	1·8

nitrogen fraction derived from the ammonia of the medium, assuming that no lysine was converted into ammonia during growth. It can be calculated that at least one-third of the lysine of the medium was incorporated into cell protein, either as such or as other amino acids, etc. Since there had obviously been metabolism of the lysine of the medium, it is probable that its final concentration was very low, though it was not found possible to detect lysine at such a low concentration in a high-glucose, high-ammonia medium; it is hence possible that the small amount of ammonia nitrogen incorporated into the cell lysine was due to the virtual exhaustion of the lysine in the medium. The pattern of distribution of ^{15}N in these cells is almost the reverse of that found in Expts. I and II, that is, the ^{15}N abundance is lowest in aspartic and glutamic acid nitrogen and in amide nitrogen, and high in tyrosine nitrogen and neutral amino acid nitrogen; this is consistent with lysine nitrogen entering the nitrogen pool of the cells by a route involving aspartic or glutamic acid in an early stage, possibly followed by amide nitrogen.

DISCUSSION

The close similarity in actual behaviour towards the components of the culture medium of two micro-organisms of such widely differing minimal nutritional requirements is striking. The only major difference appears to be in the amide groups, which in *Ln. mesenteroides* were nearly in equilibrium with the external ammonia, whereas in 9711 much unlabelled nitrogen had been incorporated; possibly *Ln. mesenteroides* was more restricted in the sources of amide nitrogen available to it. The utilization of preformed amino acid in preference to synthesis was, with lysine and organism 9711, very nearly absolute; in a medium containing 96% of its nitrogen as ammonia, about one-sixth of the total nitrogen was used for growth, yet not only was the preformed lysine used almost exclusively in the formation of cell lysine, but it was also used to some extent in the synthesis of other amino acids; despite this, the addition of this amount of lysine to the culture medium made no apparent difference to either the rate of growth of the organism or to the total amount of cell material finally synthesized.

Previous work on the utilization of peptides by bacteria deals mainly with the response of nutritionally exacting organisms to peptides containing an amino acid essential to their growth [see, for example, Fruton & Simmonds (1949), Waelsch (1952) and Kihara & Snell (1955)], and to this the finding mentioned above that *Ln. mesenteroides*, known to be exacting towards lysine, utilizes lysine peptides, is completely analogous; indeed, it is probably little more than chance that led Pader *et al.* (1948) to use *Streptococcus faecalis* rather than *Ln. mesenteroides* as test organism for the estimation of lysine in partial protein hydrolysates. The finding that a coliform organism, capable of synthesizing all its amino acids from ammonia, also utilizes lysine peptides as well as free lysine is a little more unexpected, and suggests that the phenomenon is a much more general one than had been supposed. Perhaps the hypothesis, recently re-stated by Fisher (1954), that peptides rather than amino acids are the 'normal currency' of protein metabolism, that much of the absorption and metabolism of amino acids occurs when they are conjugated as peptides, applies to micro-organisms in appropriate circumstances.

SUMMARY

1. *Leuconostoc mesenteroides* P. 60, needing 18 amino acids for growth, and a coliform organism, N.C.T.C. 9711, shown to grow well with either ammonia or peptone, etc., as nitrogen source, were grown in a medium containing salts, yeast and meat extracts, bacteriological peptone and added L-[^{14}C]lysine and [^{15}N]ammonia, and the synthesized proteins were isolated, hydrolysed and analysed.

2. The ^{15}N abundance was very high in the amide nitrogen, particularly of *Ln. mesenteroides*, and several amino acids had a low content of ^{15}N, but none was found in lysine.

3. The [^{14}C]lysine was incorporated at a level of radioactivity lower than that of free lysine in the medium, suggesting that both bacteria utilized peptide in addition to free lysine.

4. When the coliform organism was grown in a medium containing salts, glucose, lysine and [^{15}N]-ammonia only, a very small amount of ^{15}N was incorporated into the lysine of its cell protein.

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A Possible Role of Pantothenic Acid in Diabetic Cholesterologenesis

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The mechanism of cholesterol synthesis in diabetes has been studied by Hotta & Chaikoff (1952), who reported a tenfold increase in the conversion of labelled acetate into cholesterol in liver slices from alloxan-diabetic rats. The investigation of Brady & Gurin (1950) shows that in diabetes lack of insulin prevents conversion of the short-chain into long-chain fatty acids and therefore results in accumulation of the short-chain acids as the fat depots become depleted. Their investigation indicates that the accumulation of these substances accentuates the formation of cholesterol in diabetes as an alternative path of acetate metabolism. The relationship between the capacity of tissues to form acetoacetate and cholesterol synthesis has also been stressed (Gurin & Brady, 1951). Another observation (Van Bruggen, Yamada, Huchens & West, 1954), however, shows that although in the intact rat fatty acid synthesis from [1-^{14}C]acetate is impared in alloxan diabetes, the incorporation of labelled acetate into liver, gut, carcass and skin cholesterol is unchanged, suggesting unaltered cholesterologenesis.

Our previous findings indicate a relationship between decreased oxidation of acetate and acetoacetate and increased cholesterol synthesis in hypothyroïdism (Mookerjea & Sadhu, 1954*a*, 1955*b*), nephrosis (Sadhu & Mookerjea, 1955) and chronic poisoning by malonate and arsenite (Mookerjea & Sadhu, 1955*c*), but similar studies in diabetes (Mookerjea & Sadhu, 1956) appear to show a less clear picture. There is a small rise of cholesterol only in a prolonged state of diabetes of 6–7 weeks. Liver slices, however, show decreased oxidation of acetate and a considerable increase in ketogenesis. This, together with ketonuria and ketonaemia, the characteristic features of diabetes, shows that the availability of acetate and acetoacetate, the potential precursors of cholesterol, is increased in diabetes, but the less obvious rise of cholesterol indicates that they are not completely used for cholesterol synthesis.

The role of pantothenic acid in cholesterol synthesis is well established. Pantothenic acid deficiency has been shown to cause a decrease in tissue cholesterol due to lack of coenzyme A (Klein & Lipmann, 1953; Mookerjea & Sadhu, 1954*b*). The interesting observations by Migicovsky & Greenberg (1954) show the importance of relative requirement of both acetate and coenzyme A for cholesterol synthesis. They have shown that even in small amounts coenzyme A behaves as an inhibitor of cholesterol synthesis in the presence of relatively low acetate concentrations. When the acetate concentration is raised, the same amount of coenzyme A increases cholesterol formation. It appears from this that both these factors, namely availability of coenzyme A and size of acetate pool, are necessary for the synthesis of cholesterol.

The observations of Ralli & Dumm (1953), showing a great rise of adrenal cholesterol when excess of pantothenate is fed, further prompted us to investigate the importance of adequate levels of pantothenic acid for diabetic cholesterologenesis.

EXPERIMENTAL

Forty-eight male albino rats weighing 100–136 g. were fed with stock laboratory diet containing 70% of whole-wheat flour, 17% of whole-milk powder, 6% of groundnut oil, 2·5% of dried brewer's yeast, 2·5% of crude casein and 2% of salt mixture. Two drops of cod-liver oil was fed to each animal twice weekly. The average food intake/day/animal

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was approximately 7–10 g., which did not show any marked variation during the course of the experiment.

Animals of series I served as controls. Series II animals were fed daily with excess of calcium pantothenate (4 mg./10 g. of the stock laboratory diet). Series III and IV animals were rendered diabetic by the subcutaneous injection of alloxan, 200 mg./kg. body wt. Series III remained as control diabetic animals, and the diabetic animals of series IV were fed with excess of pantothenate at a similar rate to series II animals.

Excretion of sugar (Benedict, 1911) and of acetoacetate (Edson, 1935) in urine was followed during the course of the experiment. Changes in body weights of the animals were also noted.

Animals were killed after the experiment had continued for 6–7 weeks. Free and ester cholesterol of different tissues were determined according to the method of Sobel & Mayer (1945), as modified by Mookerjea & Sadhu (1955*a*) for tissue estimations.

RESULTS

Series II animals showed a greater increase in body weight than the animals of series I (Fig. 1). The fall of body weight due to prolonged diabetes (series III animals) was counteracted by excess of dietary pantothenate (series IV).

Excretion of sugar and acetoacetate was absent in series I and II animals. The sugar-excretion curves of series III and IV animals are similar. Series III animals excreted considerable amounts of acetoacetate. In the latter part of the experiment the curve shows a slight decline, while series IV animals show a sharp decline of acetoacetate excretion after an initial phase of high excretion.

Table 1 shows a definite rise of cholesterol in liver and adrenal tissues of series II animals compared with the controls (series I). The ester fraction increased more than the free cholesterol.

The cholesterol values of series III showed a similar rise to that obtained before (Mookerjea & Sadhu, 1956). The ester fractions of kidney, liver, adrenal and blood of the diabetic animals show a definite increase and the free fractions of liver and blood show a small rise.

Both free and ester cholesterol values in liver, adrenals and blood of series IV animals show a

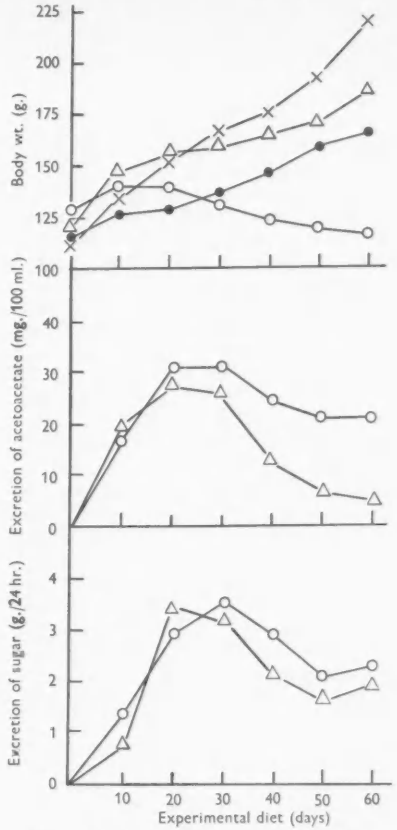

Fig. 1. Growth curve and 24 hr. excretion of sugar and acetoacetate. ●, Series I (control); ×, series II (fed on excess of pantothenate); ○, series III (diabetic); △, series IV (diabetic fed on excess of pantothenate). Each point on the curve represents the average value of the twelve animals in each group. Excretion of sugar was absent or negligible in series I and II. Excretion of acetoacetate was absent in series I and II.

Table 1. *Effect of excess of dietary pantothenic acid on the level of cholesterol in blood and tissues of normal and diabetic rats*

Average values of free and ester cholesterol in blood, liver, kidney and adrenal in mg./100 ml. or 100 g. wet wt. ± S.E. Each group consisted of twelve animals.

	Cholesterol							
	Blood		Liver		Kidney		Adrenal	
Group	Free	Ester	Free	Ester	Free	Ester	Free	Ester
I (Control)	46±3	60±4	232±8	84±7	354±12	98±4	4987±54	1082±22
II (Fed on excess of pantothenate)	50±4	62±7	248±9	128±5	350±14	102±8	6849±62	2123±32
III (Diabetic)	58±6	88±4	282±13	122±8	360±16	124±11	5247±52	1214±34
IV (Diabetic, fed on excess of pantothenate)	68±8	108±12	338±16	145±9	332±18	104±12	7456±84	2524±42

pronounced rise in these animals, compared with the animals of series III. Kidney tissue does not show any definite rise, but free cholesterol shows a small fall.

DISCUSSION

The present study attempts to elaborate a possible role of pantothenic acid in diabetic cholesterologenesis. In the liver of rats deficient in pantothenic acid, Klein & Lipmann (1953) showed a parallelism between the fall of coenzyme A level and decline of cholesterol synthesis. It appears from the study of Migicovsky & Greenberg (1954) that coenzyme A behaves as an activating factor for cholesterol synthesis, provided that the proper concentrations of acetate and cofactor are present. In untreated diabetes, when fatty acid synthesis is impaired (Brady & Gurin, 1950), unoxidized acetate and over-produced acetoacetate partially take the route of cholesterol synthesis. Their use in this metabolic pathway is, however, incomplete, as indicated by the less-pronounced rise of cholesterol in diabetes (Mookerjea & Sadhu, 1956) compared with the increased excretion of acetoacetate.

If an adequate concentration of coenzyme A is made available in diabetes, synthesis of cholesterol from its potential precursors may be further accelerated. This may be a plausible explanation for the results obtained in the present study. Animals of series IV, where excess of pantothenic acid feeding has been superimposed on diabetes, show a very sharp rise of cholesterol compared with the rise of cholesterol in animals which are either only diabetic (series III) or fed only with excess of pantothenate (series II).

The excretion of acetoacetate by series IV animals shows a sharp decline after a preliminary rise. This fact lends further support to our interpretation. Feeding of excess of pantothenic acid possibly diverts the acetoacetate which would otherwise be excreted towards further cholesterol synthesis.

Another conclusion from the present study is that the weight loss in diabetes is prevented by feeding excess of pantothenate. This fact may stimulate further research on the role and importance of pantothenic acid in diabetes.

SUMMARY

1. Free and ester cholesterol were determined in different tissues of normal rats (series I), rats fed with excess of pantothenate (series II), diabetic rats (series III) and diabetic rats fed on excess of pantothenate (series IV).

2. Series II and III animals showed small rises of cholesterol in some tissues. These rises are additive, as shown by the cholesterol levels in tissues of animals in series IV, where excess of pantothenate feeding was superimposed on diabetes.

3. Sugar and acetoacetate excretion by animals of series IV declined sharply after a preliminary rise. The growth curves of the animals also show that feeding of pantothenate prevents the weight loss of diabetic animals.

4. The availability of adequate amounts of pantothenic acid possibly guides the metabolism of unoxidized acetate and over-produced acetoacetate towards increased synthesis of cholesterol in diabetes.

5. The importance of both the availability of coenzyme A and size of acetate pool for the synthesis of cholesterol is discussed.

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Studies on the Biosynthesis of Riboflavin

3. THE UTILIZATION OF ^{14}C-LABELLED SERINE FOR RIBOFLAVIN BIOSYNTHESIS BY *EREMOTHECIUM ASHBYII**

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Considerable evidence now exists indicating that the biosynthetic routes of rings B and C of riboflavin in the moulds *Eremothecium ashbyii* and *Ashbya gossypii* are closely related to that of the purines. For example, the pattern of labelling of the carbon atoms of rings B and C and of the purine molecule is identical after the addition of $H^{14}CO_2H$, $NaH^{14}CO_3$ and [*carboxy*-^{14}C]glycine and [α-^{14}C]-glycine (Plaut, 1954). Furthermore, the addition of purines to the culture medium of *E. ashbyii* stimulates flavinogenesis very considerably (Maclaren, 1952; Goodwin & Pendlington, 1954) and this is due to its incorporation, after the removal of $C_{(8)}$, into the riboflavin molecule (McNutt, 1954).

Although glycine is incorporated into riboflavin (Plaut, 1954), it does not specifically stimulate flavin synthesis in *E. ashbyii* under conditions where serine is active (Goodwin & Pendlington, 1954). The only other amino acid to behave in the same manner as serine is threonine; tyrosine, but not phenylalanine, is very slightly active. As serine cannot be replaced by glycine plus formate (Brown *et al.* 1955) and, as the stimulatory effects of serine and purines were cumulative, it was suggested that serine might provide carbon units for synthesis of the aromatic ring A (Goodwin & Pendlington, 1954).

The present paper reports an investigation designed to test this suggestion; it is concerned with the incorporation of carbon from uniformly carbon-labelled serine and from DL-[β-^{14}C]serine into riboflavin by *E. ashbyii*.

EXPERIMENTAL

Cultures and cultural conditions. The strain of *E. ashbyii* used was kindly provided by Mrs J. Robson (A.E.R.E., Harwell, Berks). It produced more riboflavin than our original strain (Goodwin & Pendlington, 1954; Brown *et al.* 1955): otherwise it behaves similarly in that flavinogenesis is stimulated by serine and by adenine.

The medium used was that of Goodwin & Pendlington (1954), 1 l. containing: glucose, 10 g.; $CaCl_2$, 0·2 g.; $MgSO_4,7H_2O$, 1·0 g.; NaCl, 0·2 g.; bacteriological peptone, 0·4 g.

Cultures were grown at 28° in Roux bottles, generally four for each experiment, each containing 100 ml. of medium. They were connected together in series so that CO_2-free air could be drawn through continually, and the metabolic CO_2 was removed and trapped in KOH. The labelled serine was added, without dilution, in aqueous solution 48 hr. after inoculation, and the mould harvested 48 hr. later. After centrifuging off the cells and removing the riboflavin-containing medium, the residual riboflavin in the cells [30–40% of the total under our conditions (Goodwin & Pendlington, 1954)] was quantitatively recovered by adding a small volume of 2N-HCl and autoclaving at 15 lb./in.^{2} for 10 min. The two riboflavin extracts were combined and assayed spectrophotometrically (Goodwin & Pendlington, 1954). The greater part was then reduced to dryness under vacuum and the residue dissolved in 0·5N-NaOH (40 ml.). This solution was placed in a shallow glass dish so that the depth of liquid was about 1·5 cm., and the riboflavin converted into lumiflavin by irradiation with an unfiltered mercury-arc source (Hanovia, 350 w) placed at a distance of 15 cm. Conversion was generally complete within 8 hr., and this could be checked by taking a small sample, acidifying with 2N-HCl, and shaking with an equal volume of $CHCl_3$; as lumiflavin is transferred quantitatively to the $CHCl_3$ phase, the reaction is judged complete when the water phase is colourless.

In some experiments a small proportion of the medium was reduced to about one-sixth of its volume, the pH adjusted to 5·0 and $Na_2S_2O_4$ added until the E_h (redox potential) of the medium was reduced to +0·05 v (Hines, 1945; McMillan, 1945). After standing overnight the precipitated reduced riboflavin was centrifuged off and extracted with warm (60–90°) 75% (v/v) aqueous propan-2-ol; during this extraction the riboflavin was oxidized to its original state. After concentration the riboflavin was chromatographed twice on paper with butanol–water–acetic acid (4:5:1, by vol.) as solvent system. Its activity was measured after each chromatography and gave concordant results; the crude riboflavin precipitated by $Na_2S_2O_4$ always gave much higher values.

The amount of lumiflavin produced from the main portion of riboflavin was assayed spectrophotometrically (Goodwin & Pendlington, 1954), the $CHCl_3$ removed and a known weight of unlabelled lumiflavin added, the amount being usually about five times greater than the amount of labelled material so that a final weight of 10–15 mg. was obtained. Weighed amounts of this were taken for ^{14}C assay. The homogeneity of the lumiflavin was checked by paper chromatography with the butanol–water–acetic acid solvent system.

* Part 2: Brown, Goodwin & Pendlington (1955).

One portion of the lumiflavin was converted into the keto acid 1:2-dihydro-1:6:7-trimethyl-2-oxo-3-quinoxalinecarboxylic acid by dissolving in 2N-NaOH and heating for 30 min. at 100° (Kuhn & Rudy, 1934; Plaut, 1954) and the urea so formed was precipitated as dixanthylurea (Fosse, 1914). The crystalline dixanthylurea was removed by centrifuging, washed repeatedly with absolute ethanol and then water, and dried and weighed. Weighed portions were then taken for ^{14}C assay. The residual solution containing the keto acid was acidified with glacial acetic acid and the keto acid extracted with $CHCl_3$. The solvent was removed and the acid sublimed in a microsublimation tube in a stream of N_2; the CO_2 produced was thus swept out of the apparatus, trapped in $Ba(OH)_2$ (Plaut, 1954) and counted as $BaCO_3$.

The second portion of lumiflavin was converted into *N*-methyl-4:5-dimethyl-*o*-phenylenediamine by the method developed by Plaut (1954). Lumiflavin (5 mg.) was dissolved in 5N-NaOH (5 ml.) and heated in a stainless-steel bomb for 10 hr. at 150°. After cooling to room temp., the contents of the bomb were extracted with ether, the extract was dried over KOH for 3–4 hr. and the ether removed *in vacuo*. The residue was then dried *in vacuo* at 40° for 2 hr. and then dissolved in a small volume (2–4 ml.) of anhydrous ether. The diamine dihydrochloride was precipitated in a centrifuge tube (15 ml.) by passing hydrogen chloride through the solution; after centrifuging, the supernatant was removed and the precipitate washed twice with dry ether and then placed immediately in a desiccator containing soda lime and dried at 50° for 6–8 hr. (Plaut, 1954). (The dihydrochloride is an extremely deliquescent substance.) It was then weighed and counted. The complete degradation is summarized in Fig. 1.

Measurement of radioactivity. Samples were plated on stainless-steel planchets (area 2·8 cm.^{2}) and assayed with a Geiger–Müller end-window counter, working at a geometry of approximately 5%. Counts were carried to a standard error of ±5% and were corrected to zero thickness from a standard graph. In each experiment all the results are referred to the counts in the total riboflavin produced.

Radioactive materials. Uniformly labelled L-[^{14}C]serine (specific activity $5{\cdot}3 \times 10^6$ counts/min./mg.) was obained from The Radiochemical Centre, Amersham. A small amount of DL-[β-^{14}C]serine (specific activity $0{\cdot}5 \times 10^6$ counts/min./mg.) was most generously given to us by Dr E. A. Adelberg, Department of Bacteriology, University of California, Berkeley, California, U.S.A. Serine was added to the medium from an aqueous solution without dilution with unlabelled material.

RESULTS

Incorporation of labelled serine into riboflavin

Rings A, B and C. The results of two experiments, one with uniformly labelled L-[^{14}C]serine and the other with DL-[β-^{14}C]serine are recorded in Table 1.

The uniformly labelled serine gives a labelling pattern consistent with it being converted into glycine and a one-carbon fragment before incorporation into the riboflavin molecule. Considerable activity is found in $C_{(2)}$ which arises from formate (Plaut, 1954) and in $C_{(4a)}$ plus $C_{(9a)}$ which arise from glycine (it was not possible to separate $C_{(4a)}$ and

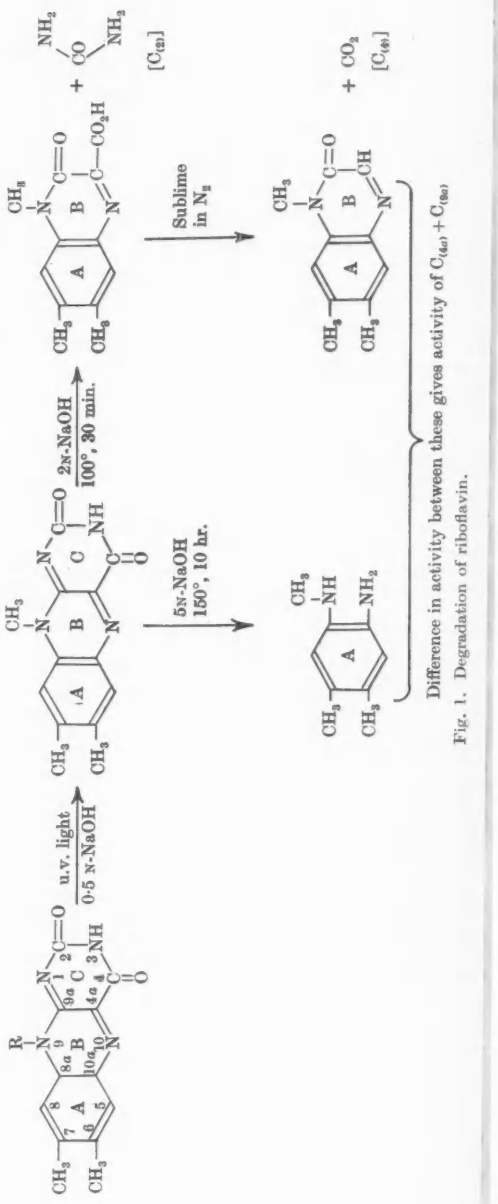

Fig. 1. Degradation of riboflavin.

Table 1. *Distribution of activity in riboflavin produced by* Eremothecium ashbyii *grown in the presence of either uniformly labelled* [^{14}C]*serine or* [β-^{14}C]*serine*

Labelled serine was added to 48 hr. cultures on basal medium of Goodwin & Pendlington (1954); cultures were harvested 48 hr. later; 100 ml. of medium was used in each Roux bottle; temp., 28°.

Compound isolated	Uniformly labelled serine: Total activity (counts/min.)	Uniformly labelled serine: Percentage of total activity incorporated in lumiflavin	[β-^{14}C]Serine: Total activity (counts/min.)	[β-^{14}C]Serine: Percentage of total activity incorporated in lumiflavin
Total counts added	2 090 000	—	105 000*	—
Lumiflavin	115 000	—	3 850	—
Dixanthylurea ($C_{(2)}$)	29 600	25·7	3 000	78
CO_2 ($C_{(4)}$)	0†	0	0	0
'Oxo-acid' ($C_{(4a)}+C_{(9a)}$)	77 000	66·9	250	6·5
N-Methyl-4:5-dimethyl-*o*-phenylenediamine (Ring A + $C_{(1)}$)	0	0	0	0
Percentage of total activity found in lumiflavin	5·5	—	3·7	—

* Twice the number of counts were added as DL-serine.
† 0 indicates that the counts were not significantly different from the background.

$C_{(9a)}$). From a consideration of the relative activities of $C_{(2)}$ and $C_{(9a)}$ plus $C_{(4a)}$, it appears that both units derived from serine are incorporated to approximately the same extent.

The greater part of the activity of [2-^{14}C]serine is found in $C_{(2)}$, indicating that the β-carbon atom of serine is a good source of 'formate'. The small but significant activity found in $C_{(4a)}$ plus $C_{(9a)}$ indicates that the one carbon unit arising from [2-^{14}C]serine can to some extent be incorporated into glycine.

In neither case is there any detectable activity in the aromatic ring A, and thus the suggestion that serine stimulates flavinogenesis by providing a carbon precursor for this aromatic ring must be discarded.

The ribitol side chain. A number of experiments (Table 2) have shown that after addition of uniformly labelled serine, the activity of the resulting riboflavin and lumiflavin are not significantly different. Thus it appears that serine contributes no carbon atoms to positions 2′, 3′, 4′ and 5′ of the ribityl residue. As the inactivity of N-methyl-5:6-dimethyl-*o*-phenylenediamine (Table 1) shows that serine is also not incorporated in $C_{(1')}$, it can be concluded that serine metabolites play no part in ribitol formation.

It is noteworthy that the incorporation of serine (4–7%) into riboflavin is much greater than that previously recorded (0·01%) for acetate (Klungsøyr, 1954). Plaut (1954) did not record the percentage incorporation of the various labelled compounds used in his experiments.

Dilution out of serine by adenine. Our first experiments with labelled serine were carried out in media containing adenine to increase the yield of riboflavin. It was soon apparent that under these conditions adenine was almost completely diluting out the serine. Table 3 demonstrates the very marked reduction in incorporation of uniformly labelled serine into riboflavin in the presence of adenine. Similar results were obtained with [β-^{14}C]serine; because of the low initial activity of the added serine, the counts in the isolated riboflavin were not significantly different from the background counts.

Table 2. *Incorporation of uniformly labelled serine into riboflavin and lumiflavin by* E. ashbyii

Riboflavin was isolated 48 hr. after addition of labelled serine to 48 hr. cultures; 100 ml. of basal medium was used in each Roux bottle; temp., 28°. Determinations on two portions of the same riboflavin sample were made in each experiment.

Total activity added	Total activity in riboflavin (counts/min.)	Total activity in lumiflavin (counts/min.)	Incorporation into riboflavin (%)
308 000	20 900	19 850	6·8
	21 200	20 500	6·9
2 080 000	96 600	112 000	4·7
	113 000	118 000	5·5

Production of carbon dioxide from serine. Table 4 demonstrates that, over 48 hr. after addition of uniformly labelled serine, 13% is metabolized to CO_2 and, after addition of [β-^{14}C]serine, approximately 7% is recovered as CO_2. This latter value is calculated by assuming that D-serine is metabolically inert. This is based on the observation that although L-threonine stimulates flavinogenesis in a similar way to serine, D-threonine is inactive (Goodwin & Pendlington, 1954). If this assumption is accepted, the results obtained suggest that *E. ashbyii* converts only two serine carbon atoms into CO_2. It will also be seen that the production of CO_2 from serine is not affected by the presence of adenine.

Fig. 1. Degradation of riboflavin.

Table 3. *Activity of riboflavin produced by* E. ashbyii *from labelled serine in the presence and absence of adenine*

Riboflavin was isolated 48 hr. after addition of labelled serine to 48 hr. cultures; 100 ml. of medium was used in each Roux bottle; temp., 28°.

	Total activity added (counts/min.)	Total activity in riboflavin (counts/min.)	Incorporation (%)
Basal medium	308 000	15 000	4·8
Basal medium + adenine (50 mg./100 ml.)	308 000	280	0·09

Table 4. *Activity of* CO_2 *produced by* E. ashbyii *from labelled serine in the presence and absence of adenine*

Riboflavin was isolated 48 hr. after addition of labelled serine to 48 hr. cultures; 100 ml. of medium was used in each Roux bottle; temp., 28°.

	Total counts added (in 200 ml. of medium) (counts/min.)	Total activity in CO_2 (counts/min.)	Percentage of total counts added in CO_2
[β-^{14}C]Serine*			
Basal medium	105 000	7 850	7·5
Basal medium + adenine	146 000	9 550	6·4
(50 mg./100 ml.)	76 000	6 040	7·8
Uniformly labelled serine			
Basal medium	2 080 000	274 000	13·2
Basal medium + adenine (50 mg./100 ml.)	817 000	103 000	12·6

* Twice as many counts added as DL-compound; it is assumed that D-serine is not converted into CO_2 (see text).

DISCUSSION

The reason why serine, but not glycine or glycine plus formate, is stimulatory appears from the present results to be its greater ease of conversion into active glycine or active formate. As both formate and glycine can be incorporated into riboflavin (Plaut, 1954), but as neither is stimulatory, either alone or in combination (Goodwin & Pendlington, 1954; Brown *et al.* 1955), and as the methylene carbon of glycine is not incorporated into $C_{(2)}$ of riboflavin, i.e. is not converted into formate (Plaut, 1954), it is possible that active formate is the limiting factor. Other situations are known in which serine is more effective than glycine; for example, (*a*) formate is essential for optimum conversion of pteroylglutamic acid into the citrovorum factor in *Streptococcus faecalis*; it can be replaced by serine but not by glycine (Broquist, Kohler, Hutchison & Burchenal, 1953), and (*b*) serine but not glycine causes a rapid disappearance of 5-amino-4-imidazole-carboxamide ribonucleotide (i.e. conversion into inosinic acid) in pigeon-liver homogenates (Buchanan, Levenberg, Flaks & Gladner, 1955).

The marked diluting out of chemically minute amounts of serine by adenine is explicable on the assumption of serine being incorporated into riboflavin via an adenine-like pool. The cumulative effect of adenine and serine when present in comparable amounts (Goodwin & Pendlington, 1954) can be due to the fact that formate, the limiting factor for purine-precursor synthesis in the basal medium, is supplied by serine whilst adenine provides extra purine precursor *in toto*. It must be assumed that units for synthesis of the aromatic ring and the ribityl side chain are not limiting.

SUMMARY

1. Uniformly labelled [^{14}C]serine is incorporated into riboflavin by *Eremothecium ashbyii* to the extent of about 4–7%; incorporation is confined to positions 2 and 4*a* plus 9*a*; no activity was found in the aromatic ring or the ribityl side chain. About 13% of the label appears as carbon dioxide.

2. [2-^{14}C]Serine is incorporated almost exclusively into position 2; a small but definite labelling occurs in positions 4*a* plus 9*a* but none in the aromatic ring. About 7% of the label appears as carbon dioxide.

3. This isotope distribution is consistent with serine being converted into glycine and formate before incorporation into riboflavin.

4. Adenine dilutes out markedly the incorporation of labelled serine into riboflavin, but has no effect on the amount of serine metabolized to carbon dioxide.

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Biological Synthesis of Ascorbic Acid: the Conversion of Derivatives of D-Galacturonic Acid into L-Ascorbic Acid by Plant Extracts

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It was suggested by Isherwood, Chen & Mapson (1953, 1954) that the synthesis of L-ascorbic acid *in vivo* can be represented in outline as proceeding by one or both of the following routes:

D-glucose → D-glucurono-γ-lactone → L-gulono-γ-lactone → L-ascorbic acid (1)

D-galactose → methyl D-galacturonate → L-galactono-γ-lactone → L-ascorbic acid. (2)

In route (2), the third stage in the synthesis, namely the conversion of the lactone of L-galactonic acid into L-ascorbic acid, has been shown to occur *in vitro* in the presence of enzymes contained within mitochondria prepared from plant tissues (Mapson, Isherwood & Chen, 1954). The corresponding reaction in route (1), namely the conversion of L-gulono-γ-lactone into L-ascorbic acid, was catalysed only to a negligible extent by the same extracts, and it is only recently that we have been able to prepare sufficiently active enzyme preparations for this reaction to be detected. The greater facility with which the last reaction in route (2) as compared with that in route (1) proceeded in the pea extracts led us to concentrate on the enzymic reaction involved in route (2) to the exclusion of (1).

In the present work, the second stage in the synthesis, namely the enzymic conversion *in vitro* of derivatives of D-galacturonic acid into derivatives of L-galactonic acid has been investigated and the enzyme mechanism associated with the reduction of the uronic acid characterized.

METHODS

Enzyme preparations

These were prepared from pea seeds which had been soaked for 24 hr. in water at 20° and germinated for a further 24 hr. at the same temperature. To reduce bacterial infection, the dry pea seeds were immersed in 1% (w/v) $HgCl_2$ for 1 min. and then thoroughly rinsed in sterile water. The partly germinated peas (30 g.) were ground up with a mixture of sand and 40 ml. of a solution of sucrose (0·4 M), phosphate 0·1 M, pH 7·4, and 4 mM-Mg^{2+} at 0°. The brei was centrifuged at 500 *g* for 5 min. and the supernatant removed. This supernatant, designated as homogenate, was further separated by centrifuging at 20 000 *g* for 20 min. into mitochondria and soluble fraction, the latter containing the enzymes present in the soluble part of the cytoplasm.

In some experiments these preparations were fractionated further by precipitation with acetone at a low temperature, as described by Askonas (1951), or by fractionation with $(NH_4)_2SO_4$. The enzyme was precipitated by acetone between 30 and 70% (v/v), and by $(NH_4)_2SO_4$ in a concentration between 50 and 70% at neutral pH.

Chemicals

Methyl D-galacturonate (MDG) and the γ-lactones of L-galactonic, D-glucuronic and D-mannuronic acids were prepared as described by Isherwood *et al.* (1954), and the amide of galacturonic acid was prepared by the method of Isbell & Frush (1950). MDG labelled with ^{14}C in the methyl group was prepared by a micro-adaptation of the usual method, with [^{14}C]methanol. When this preparation was used with the pea enzyme, the derivatives formed were examined on a paper chromatogram by means of a Geiger counter.

n-Propyl D-galacturonate was prepared under conditions similar to those used for the methyl ester, substituting *n*-propanol for methanol. Owing to the relative insolubility of galacturonic acid in *n*-propanol the yields were small, and the crude material was not purified. Chromatographic examination of the syrup showed that it consisted, to the extent of at least 85%, of a fast-running compound, presumably the *n*-propyl ester, which on saponification with alkali gave only D-galacturonic acid.

The lactone of galactaric acid was prepared by dissolving 30 g. of the free acid in 2 l. of water, boiling for 20–30 min. until a clear solution resulted, and then evaporating the solution over a free flame until the volume was reduced to

300 ml. The precipitated galactaric acid was filtered off (about 8 g. was recovered) and the filtrate evaporated *in vacuo* (bath temp. 40°) to a thin syrup. A little galactaric acid crystallized out and was filtered off. The syrup was treated with acetone, filtered from any galactaric acid which was precipitated, and the filtrate concentrated *in vacuo* to a thick clear syrup. This syrup crystallized on standing, yielding galactaric acid monolactone, m.p. 128°. Titrated with 0·1 N-NaOH at 0°, 0·191 g. of lactone consumed 9·7 ml. of alkali and at 100°, 19·1 ml. (Found: C, 37·4; H, 4·3. Calc. for monolactone of galactaric acid, $C_6H_8O_7$, 10·0 ml. and 20·0 ml. of alkali; C, 37·6; H, 4·2%.)

The solutions of DL-*iso*citrate were prepared from commercial samples of the lactone by refluxing with the equivalent amount of N-NaOH for 30 min.

The solutions of DL-malate were prepared from commercial samples of the acid recrystallized once from water.

The diphosphopyridine nucleotide (DPN) was purchased from L. Light and Co., Colnbrook, Bucks, and was stated to contain not less than 90% of DPN. It was free from triphosphopyridine nucleotide (enzymic test of Mapson & Goddard, 1951).

Triphosphopyridine nucleotide (TPN) was either purchased from Sigma Chemical Corporation, U.S.A (contained 65–80% of TPN) or prepared by a slight modification of the method of LePage & Mueller (1949). Instead of direct extraction of the liver with boiling water, it was first minced directly into five times its volume of acetone. The solid matter was filtered off, dried, ground and extracted with boiling water containing 0·5% of nicotinamide. The extracted nucleotides were then purified by adsorption on charcoal as described by LePage & Mueller. The product contained 40–60% of TPN. There was no difference in the results observed when these different preparations of TPN were used.

Paper chromatography

The apparatus and procedure were essentially the same as those described by Isherwood *et al.* (1954). Sugars and acids were detected by spraying with 0·1 M-$AgNO_3$ in 4 N aqueous NH_3 and then heating at 100° for 10 min. and those derivatives containing an aldehyde group were detected by spraying with aniline oxalate (Partridge, 1949). Esters and lactones were detected as the corresponding coloured Fe^{3+} hydroxamic acids by the method of Abdel-Akher & Smith (1951).

The sugar acid derivatives were separated with either *n*-butanol–acetic acid–water (3:1:1, by vol.) or *n*-butanol–ethanol–water (5:1:4, by vol.). The R_F values of methyl D-galacturonate and the free acid, and of the γ-lactone and free acid of L-galactonic acid, were 0·59, 0·33, 0·59, 0·37 and 0·28, 0·10, 0·34, 0·12, respectively, in the two solvents.

The examination of a particular enzyme digest varied slightly, according to the buffers and other compounds present, but a typical procedure was as follows:

The enzyme digests were acidified with H_2SO_4 to pH 2·0, centrifuged and then evaporated *in vacuo* (bath temp. 40°) to a thin syrup. Absolute ethanol (20 vol.) was added, the mixture centrifuged and the supernatant evaporated *in vacuo* to dryness. The residue was taken up in the minimum quantity of water and examined on a paper chromatogram. In certain experiments the sugar derivatives were eluted from this chromatogram and re-examined with a different solvent.

Estimation of galactonic acid derivatives formed from MDG by enzyme digests

It will be shown later that the enzyme responsible for the formation of the galactonic acid derivative is confined to the soluble part of the cytoplasm. Hence, when this enzyme had been inactivated by heating for 2 min. at 100°, the amount of the galactonic acid derivative which had been formed could be estimated by measuring the amount of ascorbic acid produced from it on the addition of mitochondria.

Washed mitochondria from peas germinated for 2 days were prepared as described by Mapson *et al.* (1954). The washed mitochondria were suspended in 0·4 M sucrose–0·1 M phosphate, pH 7·4, 1 ml. of which solution contained the mitochondria from 7 to 8 g. of germinated peas. A portion (1 ml.) of this suspension was added to 4 ml. of the test solution and the formation of ascorbic acid, determined by titration against 2:6-dichlorophenolindophenol, was followed over a period of 2–3 hr. at 37°, the maximum amount of ascorbic acid produced being recorded. With L-galactono-γ-lactone under such experimental conditions conversion into L-ascorbic acid was approximately 60% (Mapson *et al.* 1954). Until the galactonic acid derivative formed in the experiments to be reported here has been finally characterized, it is difficult to say how accurate such a means of estimation is. For this reason, in the data recorded in the illustrations we have not attempted to introduce any correction for loss during conversion into ascorbic acid. When other substances have been added to the test solutions, the effect of these on this method of estimating the galactonic acid derivative has been separately ascertained.

RESULTS

Conversion of MDG into L-ascorbic acid by enzyme preparations from peas

When MDG was added to sucrose–phosphate extracts prepared from soaked and germinated peas (homogenate), the formation of a substance reducing 2:6-dichlorophenolindophenol was observed. This substance was identified by the chromatographic procedures of Chen, Isherwood & Mapson (1953) as L-ascorbic acid. D-Galacturonic acid was not converted into L-ascorbic acid under similar circumstances. The ability of the extract to convert methyl D-galacturonate into L-ascorbic acid was subsequently found to be dependent on the presence of both mitochondrial and soluble-enzyme fractions, for on the addition of the methyl ester to either fraction separately, no formation of ascorbic acid could be observed. The synthesis of L-ascorbic acid could, however, be restored if washed mitochondria were added to the soluble fraction, showing that no essential component had been destroyed during separation of the two fractions (Fig. 1*a*).

Subsequent experiments, the results of which are illustrated in Fig. 1*b*, showed that the soluble fraction contained a heat-labile component which was necessary for the formation of L-ascorbic acid from MDG. No formation of L-ascorbic acid from

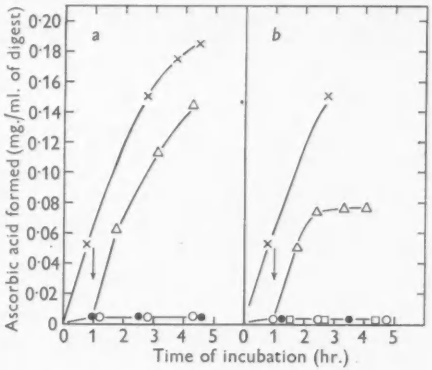

Fig. 1. Dependence of synthesis of L-ascorbic acid from methyl D-galacturonate (MDG) on separate enzymes contained in the soluble fraction of the cytoplasm and in the mitochondria. MDG was added at 3 mg./ml. to the following fractions prepared from peas and the solutions were incubated at 37°. Ascorbic acid was determined on portions as described under Methods. (*a*) ×, Homogenate; ○, mitochondria; ●, soluble fraction; △, mitochondria added to soluble fraction. (*b*) ×, Mitochondria + soluble fraction; ○, mitochondria + soluble fraction which had been inactivated by heating at 100° for 2 min.; △, soluble fraction incubated for 1 hr. with MDG, heated and mitochondria added at ↓; □, soluble fraction incubated for 1 hr. without MDG, mitochondria added at ↓; ●, soluble fraction incubated for 1 hr. with MDG, then mitochondria, previously inactivated by heat, added.

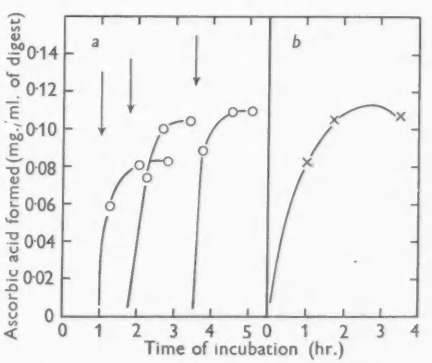

Fig. 2. Conversion of MDG, by enzymes present in the soluble fraction, into a compound which in turn is converted into L-ascorbic acid by mitochondria. (*a*) Portions of the soluble-enzyme fraction were incubated with MDG (3 mg./ml.) at 37° for 1, 1·75 and 3·5 hr., heated and mitochondria added at ↓. (*b*) The maximum amount of ascorbic acid formed in (*a*), plotted against time.

the methyl ester could be detected in the presence of washed mitochondria and a soluble fraction which had previously been heated to 100° for 2 min., although L-galactono-γ-lactone was readily converted into L-ascorbic acid by this extract, showing that the activity of the mitochondria had not been altered.

The position was made still clearer by further experiments in which MDG was incubated with the soluble fraction for varying periods of time at 37°. Enzymic activity was arrested by adjusting the pH to 5·5, heating at 100° for 2 min. and removing the coagulated protein by centrifuging. The pH of the supernatant was brought to pH 7·4, and after the addition of washed mitochondria the solution was re-incubated at 37°. Samples of the solution were then taken at intervals for the estimation of L-ascorbic acid. The results showed that L-ascorbic acid was rapidly formed on the addition of mitochondria (Fig. 2*a*) and that the amount formed increased with increase in time of incubation of the methyl ester with the soluble fraction (Fig. 2*b*).

Identification of a derivative of L-*galactonic acid as an intermediate in the conversion of MDG into* L-*ascorbic acid*

Examination on paper chromatograms of digests containing MDG and the soluble fraction of the extract from soaked peas revealed the presence of a derivative of galactonic acid. To obtain a complete separation of the various compounds present the extract was examined with the *n*-butanol–acetic acid–water solvent, and the fast-running material re-examined with *n*-butanol–ethanol–water as solvent. This chromatogram revealed the presence of a galactonic acid derivative having the same R_F value as L-galactono-γ-lactone (Fig. 3). Control experiments showed that this substance was absent if the enzymes in the extract were first inactivated by boiling before the addition of the ester. In further experiments an approximate quantitative assay of the amount of the galactonic acid derivative formed in the enzyme digest was made by comparing the size and intensity of the spot on the chromatogram with a similar chromatogram prepared from a heat-inactivated enzyme, to which both the methyl ester and an amount of L-galactono-γ-lactone had been added in concentrations equal to those estimated to have been formed in the enzyme digest. This estimate was made by determining, on a separate portion of the enzyme digest, the L-ascorbic acid formed on the addition of mitochondria. The results showed that the amount of galactonic acid formed during the reaction could account for the amount of L-ascorbic acid subsequently formed on the addition of mitochondria.

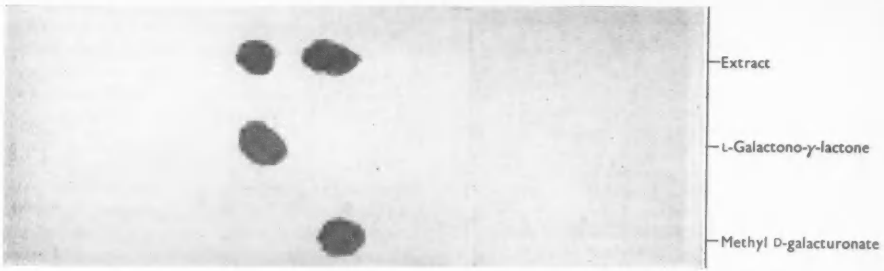

Fig. 3. Chromatogram of the galactonic acid derivative formed enzymically from MDG.

Further experiments showed that the substance formed from the methyl ester behaved very similarly to L-galactono-γ-lactone in its stability in solutions of pH varying from 6·0 to 8·0. MDG was added to the soluble-enzyme fraction and incubated at 37° for 3 hr. to allow for maximum conversion into the galactonic acid derivative. After inactivating the enzyme by heat or by bringing the pH to 4·0, the pH values of portions were adjusted to 6·2, 6·9 and 7·5, the separate solutions placed at 37°, and the concentration of the galactonic acid derivative in the solution after varying periods of time was measured by determining the amount of L-ascorbic acid formed on the addition of mitochondria. The rates of decomposition of the galactonic acid derivative formed from the methyl ester, and of L-galactono-γ-lactone, showed that both reactions were kinetically of the first order, and the values of k (rate constants) identical within the limits of error inherent in the method of estimation. Furthermore, as Fig. 4 shows, the effect of pH on the values of k was very similar in both cases.

With [*methyl*-^{14}C]MDG the galactonic acid derivative isolated on the chromatogram showed no radioactivity, indicating that this substance could not be the methyl ester of L-galactonic acid. As already shown, the galactonic acid derivative is rapidly converted into L-ascorbic acid on the addition of mitochondria, and this fact indicates that the intermediate is a derivative of L-galactonic acid, since derivatives of D-galactonic acid are not so converted (Mapson *et al.* 1954). These results strongly suggest that the L-galactono-γ-lactone is a product of the reduction of the methyl ester; they do not, of course, exclude the possibility that methyl L-galactonate may be formed first and then rapidly converted into the lactone.

The results of these and the earlier experiments show that the formation of L-ascorbic acid from methyl D-galacturonate occurs in two stages. The first stage, that of the reduction of the methyl ester to a derivative of galactonic acid, is catalysed by

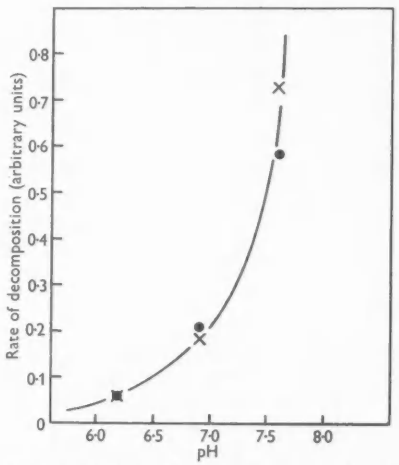

Fig. 4. Effect of pH on the rate of decomposition of the galactonic acid derivative formed from MDG (●), and L-galactono-γ-lactone (×). For details see the text.

an enzyme present in the soluble fraction but apparently absent from the mitochondria. The word 'apparently' is used here because it is conceivable that the methyl ester may not be able to reach the active enzyme centre within the mitochondria, but the galactonic acid derivative formed from it may. This is unlikely, however, in view of our previous experience of the ease of penetration of sugar lactones and esters into cells (Isherwood *et al.* 1954). The second stage in the synthesis takes place in the mitochondria and consists of the enzymic oxidation of the galactonic acid compound into L-ascorbic acid.

The reducing system

Having established that the reaction in the soluble fraction was concerned with the reduction of the methyl ester of uronic acid, the next phase in our

investigation was to study the enzyme system catalysing the reduction. The reduction of many compounds in biological systems is mediated through the agency of either DPN or TPN, and we therefore carried out experiments to see whether these coenzymes were involved.

An attempt was made, in the first instance, to separate the reducing system into its components. This was achieved by dialysis of the soluble fraction against 0·025 M phosphate buffer, pH 7·4; after 48 hr. the catalytic activity of the dialysed extract was reduced to zero. This loss of activity was not due to inactivation of enzyme, for it could be restored by adding heat-stable components present in the undialysed extract. The nature of these components was revealed in the following experiments. The presence of appreciable quantities of malic and *iso*citric acids in the undialysed extract was confirmed by chromatographic analysis (cf. Mapson & Goddard, 1951) of the acid fraction separated from the extract by means of ion-exchange resins according to the method described by Partridge (1952). However, the addition of these acids alone to the dialysed enzyme preparation did not initiate the reaction, but if they were added in addition to TPN, reduction of the methyl D-galacturonate occurred (Fig. 5). DPN could not be substituted for TPN, and the negative response with the former coenzyme was not due to the absence of DPN-linked dehydrogenases or of their substrates. Active formic and alcohol dehydrogenases specific for DPN and malic and *iso*citric enzymes specific for TPN were known to be present in extracts from ungerminated peas (Mapson & Goddard, 1951), and their presence in these extracts was confirmed. The dialysed extract also contained an active aconitase enzyme, since both citrate and *cis*-aconitate, but not *trans*-aconitate, could be substituted for *iso*citrate.

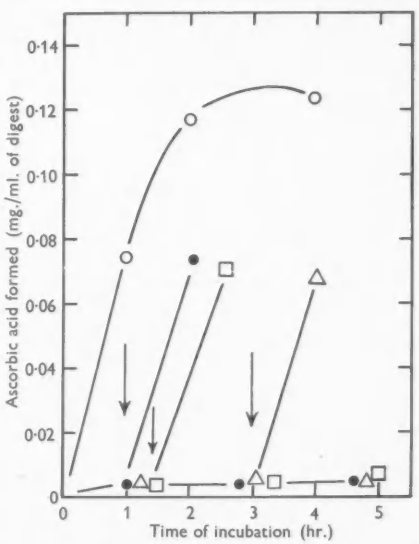

Fig. 5. TPN as coenzyme in the enzymic reduction of MDG by an enzyme in the soluble fraction of the cytoplasm of peas. ○, Complete system containing enzyme + TPN + malate + MDG; ●, enzyme + TPN + MDG (↓ malate added to a portion); □, enzyme + malate + MDG (↓ TPN added to a portion); △, enzyme + MDG (↓ malate + TPN added to a portion). The enzyme consisted of the soluble fraction dialysed against 0·1 M phosphate, pH 7·4, for 48 hr. at 0°. The various substances used were added to give a final concn. of TPN of 10 μg./ml.; MDG, 3 mg./ml.; malate, 2 mg./ml.; 4 mM-Mg^{2+} was present in all solutions; temp. 37°. The reduction product was estimated as ascorbic acid (see Methods).

Enzymic oxidation of reduced triphosphopyridine nucleotide (TPNH) by derivatives of D-*galacturonic,* D-*glucuronic and* D-*mannuronic acids*

The results of these experiments made it seem likely that the reduction of MDG occurred at the expense of TPNH, and suggested the presence of an enzyme capable of catalysing a reaction between derivatives of galacturonic acid and reduced TPN. This has been confirmed with a purified enzyme preparation which made it possible to follow the oxidation of TPNH spectrophotometrically (decrease in absorption at 340 mμ.) on the addition of the methyl ester.

The enzyme preparation used for these experiments was prepared by extracting 30 g. of peas germinated for 2 days with 40 ml. of 25 mM phosphate, pH 7·4, centrifuging at 20000 *g* for 20 min., and dialysing the supernatant against the same buffer for 24–48 hr. at 1°. The dialysed enzyme solution was further purified by removal of some protein, which was precipitated at pH 5·0–5·3 with dilute acetic acid, care being taken to keep the solution cold. This procedure was essential, not only to clarify the solution but to remove an enzyme protein which catalysed the oxidation of TPNH in the presence of oxygen. The nature of this enzyme has not been investigated but may be similar to that found by Conn, Kraemer, Pei-Nan Liu & Vennesland (1952). After centrifuging for 5 min. at 20000 *g* the pH of the clarified enzyme solution was adjusted to 6·5.

The enzyme preparation contained active *iso*citric and malic enzymes, and the reduction of added TPN could be effected simply by the addition of either *iso*citrate or malate. A portion (1 ml.) of this enzyme solution was added to 2 ml. of 0·1 M phosphate buffer, pH 7·4, containing 4 mM Mg^{2+} in a 1 cm. spectrophotometer cell; a similar solution was used as a blank. TPN (0·35 mg./ml.) was added to one cell and malate or *iso*citrate to both. These latter compounds were added only in sufficient concentration (80% of the theoretical) to effect a

partial reduction of the coenzyme. The reduction of TPN was followed by observing the increase in absorption at 340 mμ. After the reduction had ceased, the methyl ester was added (3 mg./ml.) and the oxidation of the TPNH followed by observing the decrease in optical density, a reaction which could be reversed by the addition of further malate or *iso*citrate.

The results of two experiments of this type are illustrated in Fig. 6, and show the effect of MDG under aerobic and anaerobic conditions. It will be noticed that under aerobic conditions a small but steady oxidation of TPNH, due to the presence of a residual amount of the TPN oxidase referred to above, occurred before the addition of the methyl ester, but that this reaction was prevented in the absence of oxygen.

Similar results were obtained when the *n*-propyl ester was substituted for the methyl derivative, the rate of oxidation of TPNH with the former ester being only slightly slower than that with the latter. The other derivative of galacturonic acid tested was the amide; this, however, was not reduced. The γ-lactones of the two other naturally occurring uronic acids, D-glucuronic and D-mannuronic acids, were reduced, but at slower rates than methyl D-galacturonate; the reaction rate with D-mannurono-γ-lactone was only half, and with D-glucurono-γ-lactone only slightly greater than a third, of the rate observed with the esters of galacturonic acid.

In these experiments it was observed that the rate of reduction of TPN by either the malic or *iso*-citric enzyme systems was several times greater than the enzymic oxidation of TPNH by the methyl ester, so that under conditions in which either malate or *iso*citrate is in excess of that required for the complete reduction of the ester, we would anticipate that the concentration of the reduced coenzyme would remain constant throughout the reaction. This was shown to be so in experiments similar to those described above, in which *iso*citrate was added in excess. Under these conditions there was no significant change in concentration of TPNH after the addition of the methyl ester. These results were found to be of importance in the kinetic experiments which are dealt with later in this paper.

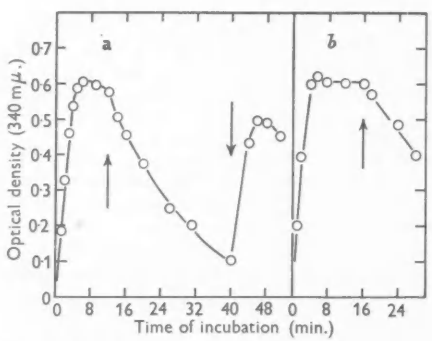

Fig. 6. Oxidation of TPNH by MDG, catalysed by an enzyme from the soluble fraction, at pH 6·9 and 17°. TPN (0·35 mg./ml.) and *iso*citrate (0·1 mg./ml.) were added at zero time; MDG (3 mg./ml.) and *iso*citrate (0·1 mg./ml.) were added later; ↑, MDG; ↓, *iso*citrate. (*a*) In air, (*b*) in nitrogen. For details of estimation see Results section.

Substrate–velocity and pH *relations*

The relation between concentration of substrate and rate of reaction of MDG is shown in Fig. 7*a*. The high Michaelis constant K_m of 10^{-2} indicates a low affinity of the enzyme for this substrate, and suggests that the methyl ester may not be the naturally occurring precursor.

The effect of pH on the enzymic reaction between the methyl ester and TPNH was measured spectrophotometrically, and the results obtained are shown in Fig. 7*b*. The optimum pH for the reaction appears to be in the region of pH 7·0–7·5. Similar results to these were obtained when catalytic amounts of TPN with the *iso*citric enzyme and its substrate were used to regenerate TPNH.

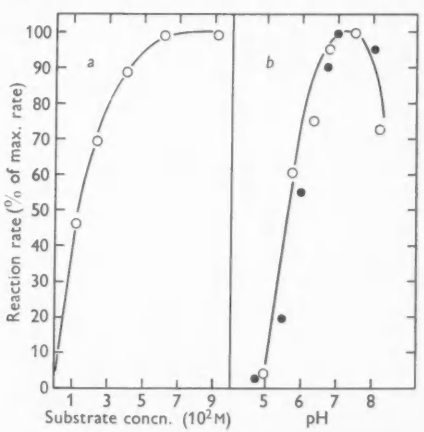

Fig. 7. Effect of concentration of substrate and of pH on the rate of reduction of MDG by an enzyme present in the soluble fraction of the cytoplasm from peas. (*a*) Velocity/substrate concn. relation, (*b*) velocity/pH relation. ○, Reaction rates at 17° determined by spectrophotometric measurement of the oxidation of TPNH (see text). ●, Reaction rates at 37° determined by the use of *iso*-citric enzyme system with TPN and measuring the reduction product by converting it into ascorbic acid with mitochondria.

Inhibitors

The effect of a number of inhibitors on the activity of the enzyme was determined. In these experiments the enzyme was prepared by the method described above, and its activity likewise determined by measuring spectrophotometrically the rate of oxidation of TPNH on addition of MDG. The substances tested for their ability to inhibit were added to the enzyme solution in 0·1 M phosphate, pH 7·0, at 20° and the assay was made after 15 min. There was little or no inhibition with fluoride, arsenite, arsenate, azide, maleate, iodobenzoate or ethylenediaminetetraacetic acid when these were used in millimolar concentration, or with 0·2 mM dinitrophenol. At millimolar concentration a slight inhibition (18%) was observed with iodoacetate, and a somewhat larger one (25%) with KCN.

Progress of the reaction

MDG is only converted into the L-galactonic acid derivative, even with enzyme extracts of high activity, to an extent of about 10%, even after allowing for the fact that about 40% of the galactonic acid derivative formed was decomposed under our experimental conditions before it could be converted into ascorbic acid by mitochondria. Examination of the progress curves during the first 3–4 hr. (Figs. 8, 9) show that the cause of this poor conversion was due to the slowing down of the reaction after about 1·5 hr. at 37°. The early termination of the reaction may be thus due to one or more of several causes. These include (1) exhaustion or destruction of substrates or coenzyme, (2) destruction or inhibition of enzymes concerned, (3) instability of reduction product, and (4) attainment of chemical equilibrium.

Consideration was first given to the possibility that an impurity in the methyl ester was the substrate actually being converted. The most likely impurity was the methyl glucoside, which is formed to the extent of 8% during the esterification of galacturonic acid by the method of Jansen & Jang (1948). The possibility was ruled out on finding that the glycoside itself gave negative results, and that recrystallization of the methyl ester gave results in which the percentage conversion was slightly higher rather than lower as would be expected if the real substrate was an impurity.

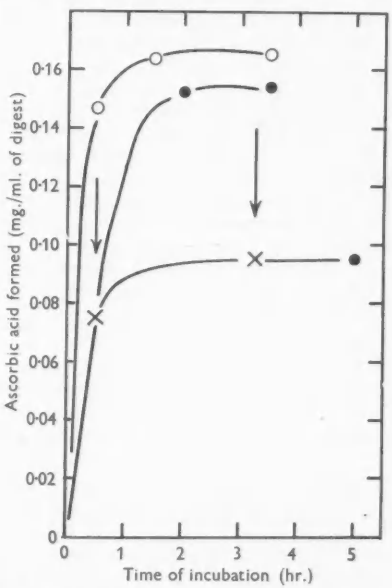

Fig. 8. Effect of altering the concn. of MDG on the progress of the conversion into L-galactono-γ-lactone (measured after conversion into ascorbic acid) at 37°. ○, Initial concn. of MDG at zero time, 12 mg./ml. of digest. ×, Initial concn. of MDG, 3 mg/ml.; ↓ ● denotes addition of further amounts of MDG, 9 mg./ml. The digest consisted of the soluble fraction from peas, exhaustively dialysed against 0·1 M phosphate, pH 7·4, together with DL-*iso*citrate (4 mg./ml.), TPN (10 μg./ml.) and Mg^{2+}, 4 mM.

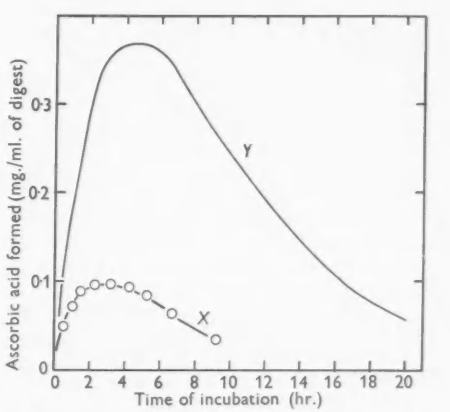

Fig. 9. A comparison of the experimental-progress curve of the enzymic reduction of MDG to L-galactono-γ-lactone with a theoretical curve based on the assumption of consecutive first-order reactions for this and the succeeding conversion into ascorbic acid (see text). *X*, Experimental curve; *Y*, theoretical curve. Experimental curve obtained by following the enzymic production of a galactonic acid derivative from MDG, the concentration of this derivative being estimated by oxidizing it to ascorbic acid with mitochondria (see Methods). Enzyme system composed of dialysed soluble fraction from peas with malate (4 mg./ml.) and TPN (10 μg./ml.) in 2·5 mM phosphate buffer, pH 7·4, at 37°.

It seems clear that the reaction does not cease because of destruction of either malate or *iso*citrate enzymes, their substrates or the coenzyme TPN. This was shown by adding these substances, either separately or collectively, to an enzyme digest in which the reaction had ceased. The addition of more malate, *iso*citrate or TPN did not prevent the fall off in the reaction. Active *iso*citrate dehydrogenase preparations from heart muscle (Grafflin & Ochoa, 1950), added with or without extra TPN, also gave negative results. Moreover, in enzyme digests which had been incubated for 4 hr. at 37° an active *iso*citric enzyme could be demonstrated by observing the reduction of added TPN spectrophotometrically.

The slowing down of the reaction could not be attributed to the disappearance of the methyl ester. Chromatograms of extracts of enzyme digests taken at times when the reaction had ceased showed that appreciable quantities of the ester were still present. Moreover, if after the reaction had slowed down the concentration of MDG was increased by an amount equal to that originally added, no significant stimulation was observed; only when MDG was added at an earlier stage was any positive effect observed, and this could be attributed simply to the effect of an increased concentration of the substrate on the velocity of the reaction (Fig. 8). These data likewise indicate that the reaction does not slow down because of the attainment of equilibrium between the methyl ester and its reduction product.

The following experiments were carried out to test whether the enzyme catalysing the transfer of electrons from TPNH to the methyl ester was being inactivated during the course of the reaction. The enzyme could be incubated at 37° for 3 hr. before the addition of any of the reactants, without its activity being diminished. The same was true if the enzyme preparation was incubated at 37° for 3 hr. either with malate or TPN or with the methyl ester. It seems clear that the enzyme was not inactivated by any one of the components of the system, and it became necessary to determine if the enzyme was inhibited by products formed during the reaction. Certainly the enzyme was not irreversibly inactivated during the reaction, for when this had ceased and the extract was dialysed overnight to remove soluble constituents, the activity of the enzyme when retested was as great as that observed originally. Nevertheless, the results illustrated in Fig. 8 are most simply interpreted on the basis that some inactivation of the enzyme occurs during the course of the reaction. This explanation is further supported by results of experiments in which it was found that, after the formation of the galactonic acid derivative stopped, addition of fresh enzyme, either in the form of an acetone-dried powder or prepared as a concentrate after fractionation with $(NH_4)_2SO_4$, led to a resumption of the reaction for a period of about 1 hr.; the reaction slowed down again subsequently.

Identification of the inhibitor with products which might be formed during the reaction has so far met with little success. No inhibition of the enzyme was detected in the presence of galacturonic acid, the methyl glycoside of the acid, methanol, galactono-γ-lactone, or galactonic acid, when these were added at a concentration equivalent to that of the methyl ester. In addition, it was shown that ascorbic acid itself did not influence the reduction of the methyl ester. Nor could the products formed by the malic or *iso*citric enzyme systems be responsible, for if these enzymes were allowed to react [e.g. by reducing added oxidized glutathione in the presence of glutathione reductase] before the addition of the methyl ester, the rate and extent of the reduction of the ester were not affected.

Galactaric acid and its lactone, which might be produced by the oxidation of MDG in the extract, both inhibited the enzyme (30 % reduction in rate at 2·5 mM concentration), but this inhibition was not increased by previous incubation with the enzyme, as would have been expected if these substances were formed during the reaction and were the agents responsible.

A factor which does operate to limit the concentration of the galactonic acid derivative formed is its instability. We have already shown that the effect of pH on the stability of this derivative was very similar to that of L-galactono-γ-lactone. Whilst a change in pH from 6·5 to 7·5 has no large effect on the rate of reduction of the methyl ester, it has a marked influence on the rate of breakdown of the reduced product, with the result that the maximal amount of this substance formed is much less at pH 7·5–8·0 than at neutral pH. In the system under investigation we have:

$$\text{MDG} + \text{TPNH} \xrightarrow{k_1} \text{galactonic acid derivative} + \text{TPN}, \quad (3)$$

$$\text{galactonic acid derivative} \xrightarrow{k_2} \text{inactive products}, \quad (4)$$

where k_1 and k_2 are the appropriate rate constants.

As noted earlier, under the conditions of these experiments, the concentration of TPNH remains constant throughout the reaction. Provided, in addition, that the activity of the enzyme catalysing the reduction of the methyl ester remains unimpaired reaction (3) will be kinetically of the first order. The decomposition of the galactonic acid derivative formed also appears to be kinetically first order as was shown earlier. For a system of consecutive first-order reactions,

$$[\text{A}] \xrightarrow{k_1} [\text{B}] \xrightarrow{k_2} [\text{C}], \quad (5)$$

the concentration of [B] (galactonic acid derivative) as a function of time (t) is given by (see Daniels, 1948):

$$[B]=\frac{k_1A_0}{2(k_2-k_1)}(e^{-k_1t}-e^{-k_2t}). \quad (6)$$

The value of k_2 was calculated from the experimental data described earlier, on the rate of decomposition of L-galactono-γ-lactone. Under similar experimental conditions k_1 was evaluated by inserting into eqn. (6) the experimental values for B taken from the early stages of the experimental curve. With these values of k_1 and k_2 a curve was constructed representing the theoretical amounts of the galactonic acid derivative which one might expect to be formed, if the reaction obeyed eqn. (5). Comparison of the theoretical curve (Y) with the experimental curve (X) (Fig. 9) clearly indicates that other factors, besides the unstable nature of the reaction product, are responsible for the poor conversion.

Influence of mitochondria on the progress of the reaction

Observations, susceptible of several interpretations, were made on the effect on the reaction of adding mitochondria. If mitochondria were added to the enzyme digest after the reaction had ceased, the reaction was restarted, as evidenced by the greater amount of ascorbic acid formed compared with a similar enzyme digest which had been inactivated by heat before the addition of mitochondria. This action of the mitochondria could not be interpreted as being due to the supply of coenzymes or of other heat-stable factors, since the addition of extracts made from mitochondria had no effect.

The effect of the mitochondria was abolished in the absence of oxygen or in its presence if azide and succinate were added, circumstances in which the conversion of galactonic acid derivatives into L-ascorbic acid are prevented by these particles (Mapson *et al.* 1954). However, if the sole action of the mitochondria was to convert the intermediates into L-ascorbic acid, thus preventing the breakdown of the former, the rate of the reaction stimulated by the addition of these particles should be equivalent to the rate of breakdown of the intermediates. In fact, the mitochondria increased the rate of reaction to a value at least four times that which would be expected from this cause alone.

The conversion of MDG into ascorbic acid in the presence of both soluble and particulate fractions proceeds smoothly for a period of 3–4 hr. at 37°, thereafter the rate of the reaction decreases fairly rapidly. The cause of this has not been investigated, but it seems probable that it is connected with changes taking place within the mitochondria.

DISCUSSION

We have shown in this paper that certain derivatives of galacturonic acid are reduced to derivatives of galactonic acid by an enzyme system present in peas. The reaction has features which require further investigation. Because we had shown previously that derivatives of galactonic acid, but not the free acid, were converted into ascorbic acid (Mapson *et al.* 1954) we were not surprised to find that the enzyme would not catalyse the reduction of free galacturonic acid, but we did anticipate that simple derivatives of galacturonic acid, in which the ionization of the carboxyl group had been suppressed, would be reduced. The reduction of the methyl and *n*-propyl esters of galacturonic acid and of the lactones of glucuronic and mannuronic acid appeared to support this hypothesis. However, the inability of the enzyme to catalyse the reduction of the amide of galacturonic acid seems to indicate that the nature of the group attached to the carboxyl is not unimportant.

The greater rate of reduction of derivatives of the parent sugar D-galactose, compared with derivatives from D-glucose, is in line with the results obtained with the aldonic acids (Mapson *et al.* 1954) and makes it more probable that derivatives of D-galactose rather than D-glucose are the natural precursors of L-ascorbic acid in plants.

The other findings, for which we have as yet no precise explanation, are the premature termination of the reaction and the role of the mitochondria in preventing this. We have examined a number of possibilities and our results indicate that, of these, inactivation of the enzyme during the reaction appears to be the chief factor, though so far we have not been able to find the cause. The low affinity of this enzyme for the methyl ester makes it probable that this simple derivative of galacturonic acid is not its natural substrate.

SUMMARY

1. The enzymic reduction of derivatives of D-galacturonic acid to derivatives of L-galactonic acid has been demonstrated in peas. The enzyme catalyses a reaction between reduced triphosphopyridine nucleotide and esters of D-galacturonic acid; the free acid is not reduced.

2. In peas which have been germinated for 2–3 days, the enzyme is located in the soluble part of the cytoplasm, and is absent from the cytoplasmic particles (mitochondria). The formation of L-ascorbic acid from derivatives of D-galacturonic acid thus requires the presence of this enzyme, and the enzyme system within the mitochondria which converts derivatives of L-galactonic acid into L-ascorbic acid.

3. Evidence from chromatographic and kinetic studies suggests that a product of the reduction of methyl D-galacturonate is L-galactono-γ-lactone,

although the prior formation of an unstable intermediate is not excluded.

4. The poor conversion of esters of D-galacturonic acid into compounds of L-galactonic acid appears to be due mainly to the reversible inhibition of the enzyme during the course of the reaction, and to a lesser extent to the instability of the galactonic acid derivative formed. The substance responsible for the inhibition of the enzyme has not been identified.

5. The low affinity of the enzyme for methyl D-galacturonate (K_m, 10^{-2}M) suggests that this is not the naturally occurring substrate.

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Further Studies on the Reactions of Disulphides with Sodium Sulphite

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In a previous paper (Cecil & McPhee, 1955*b*) the reactions of oxidized glutathione, cystine and some cystine derivatives were described. It was found that SO_3^{2-} ions react according to the equation

$$R.S.S.R + SO_3^{2-} \rightleftharpoons R.S^- + R.S.SO_3^-,$$

but that HSO_3^- ions do not react. Since the reaction with $R.S^-$ is reversible (Footner & Smiles, 1925) the equilibrium

$$R.S^- + H^+ \rightleftharpoons R.SH$$

has also to be taken into account. It was found that the negatively charged disulphides react much more slowly than do those with no net charge. Thus with oxidized glutathione and cystine there is an optimum pH at which the observed rate of reaction is maximal. Below the optimum the observed rate falls off because of decreasing SO_3^{2-} concentration, and above it because of increasing negative charge on the disulphide molecule due to dissociation of the amino groups.

This paper describes similar studies on a number of other compounds, including homocystine and two positively charged compounds, bis-(β-aminoethyl) disulphide (cystamine) and bis-(γ-aminopropyl) disulphide (homocystamine). It has been found, as was expected from the previous results, that the positively charged compounds react much more rapidly than those with no net charge. Homocystine and its derivatives react more slowly than the corresponding cystine derivatives, but otherwise show the same general behaviour. The application of this work to the disulphide groups of proteins is discussed.

The theoretical descriptions of disulphide–sulphite reaction and equilibrium mixtures have been given in detail (Cecil & McPhee, 1955*b*), and the same notation will be used here.

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EXPERIMENTAL

Reagents

Cystamine. A sample of the dihydrochloride was kindly supplied by Horlicks Ltd., Slough, Bucks. (Found: Cl, 30·7; $C_4H_{14}N_2S_2Cl_2$ requires Cl, 31·6%.) Standard solutions of the dinitrate were made by adding the calculated amount of $AgNO_3$ to solutions of the hydrochloride in 0·1 N-HNO_3, filtering off the AgCl and making the filtrates up to the required volumes.

Cystine hydantoin. This was prepared from cystine by the method of Hess (1934). Standard solutions were made up in 0·01 N-HNO_3.

Homocystine hydantoin. This was prepared from homocystine by the method of Karabinos & Szabo (1944), m.p. 201–202° (corr).

Diformylhomocystine. This was prepared from homocystine by the method described by Fruton & Clarke (1934) for diformylcystine. (Found, C, 37·2; H, 4·9; N, 8·7; $C_{10}H_{14}O_4N_4S_2$ requires C, 37·1; H, 4·9; N, 8·6%.)

Homocystamine. Homocystamine dihydrobromide was prepared by the method of Schöberl & Kawohl (private communication from Professor A. Schöberl; also Kawohl, 1953). Standard solutions of the dinitrate were made up as described for cystamine.

Methods

The other reagents used and the experimental details of the analytical method and kinetic experiments have been described previously (Cecil & McPhee, 1955*a*, *b*). Briefly, kinetic experiments were carried out in buffered solutions over the pH range 4–11·5, samples were withdrawn at measured time intervals and the reactions stopped by bringing the pH to 1·8–2·2 with HNO_3 or H_2SO_4. The thiol concentrations were then found by potentiometric titration with $AgNO_3$.

The disulphide concentrations used were between 0·4 and 1·5 mM, and the sulphite concentration was varied from approximately equimolar with disulphide to a 40-fold molar excess.

Na_2SO_3 was used as its own buffer whenever possible, i.e. when the sulphite was present in greater than 10-fold excess over disulphide, in the pH range 6·0–7·4. Other buffers used were acetate, phosphate, borate and carbonate.

Determination of dissociation constants. Samples of carbonate-free solutions of cystine hydantoin, homocystine hydantoin and cystamine were titrated potentiometrically with standard carbonate-free NaOH solution in a closed titration flask, with a Cambridge Alki glass electrode. Oxygen-free N_2 was bubbled through continually and served to keep the solution stirred. The glass electrode was standardized against a hydrogen electrode.

RESULTS

Estimation of the disulphides

The methods of estimation for homocystine and five other disulphides (by allowing them to react with Na_2SO_3 and titrating the thiol formed with $AgNO_3$) have been reported (Cecil & McPhee, 1955*a*), and methods for five more disulphides are summarized in Table 1. The corresponding thiols have not been studied as pure substances but only as disulphide–sulphite reaction mixtures. No errors were found for the titrations in acid solution of β-aminoethanethiol (cysteamine), (which was titrated in H_2SO_4, not HNO_3, as the latter medium gave flat, asymmetric titration curves), γ-aminopropanethiol (homocysteamine) or cysteine hydantoin. With homocysteine hydantoin, errors which varied slightly with pH occurred below pH 4·3. In the range of pH 1·9–2·3 the error was +3% and was constant over the range of thiol and buffer concentrations used. Similarly, *N*-formylhomocysteine gave a +1% error over the pH range 2·0–2·3. This error varied considerably with pH, rising to +10% at pH 3·2 and 8% at pH 1·75. It was unchanged in the presence of borate or carbonate. As before (Cecil & McPhee, 1955*b*), correction factors were applied to the titrations done during kinetic experiments.

Homocysteine showed unusual behaviour in acid solution, the titration error varying continuously with pH and sulphite concentration. With a 20-fold molar excess of sulphite over thiol (in solutions acidified with HNO_3), the titration error varied from +10% at pH 1·7 to +2% at pH 2·3. At pH 2·2 the error varied from 0 when the sulphite excess was less than fivefold to +12% when the excess was 100-fold. Thus a series of control titrations with homocysteine at an identical titration pH had to be carried out after each kinetic experiment to find the correction factors to be used; these varied in any one experiment since the sulphite:thiol ratio varied continuously. The errors were of the order of 3–10%, being 1–2% higher in the presence of borate or carbonate than when the salts were absent.

Dissociation constants of groups in the disulphide molecules

The dissociation constants of cystamine and homocystine hydantoin were calculated from the titration data by the method of Simms (1926). With

Table 1. *Estimation of disulphides*

Temperature of reaction was 60° for diformylhomocystine; the other compounds were allowed to react at room temp. In each case the titration with $AgNO_3$ was done at the reaction pH.

	Reaction conditions with Na_2SO_3			
Substance	Moles of Na_2SO_3/mole of disulphide	pH	Time allowed for reaction (min.)	Titres (% of weighed concn.)
Cystamine	10–20	6·5–7·0	<1	97
Homocystamine	10–20	6·5–7·0	<1	97
Cystine hydantoin	15–25	6·5–7·0	<1	100
Homocystine hydantoin	20–30	6·5–7·0	10	99
Diformylhomocystine	30–40	7·0–7·5	30	100

Table 2. *Alkaline dissociation constants of the disulphides*

	Cystamine		Homocystine*		Homocystine hydantoin	
Temp.	pK'_1	pK'_2	pK'_3	pK'_4	pK'_1	pK'_2
25°	8·6	9·5	8·5	9·4	8·7	9·7
0	9·3	10·2	9·2	10·1	9·1	10·1

* From the data of Ryklan & Schmidt (1944).

his notation, the dissociation constants at 20° of homocystine hydantoin were found to be pK'_1 8·65, pK'_2 9·68, and of cystamine pK'_1 8·69, pK'_2 9·61. The dissociation constants of cystine hydantoin could not be determined, since this compound began to decompose above about pH 9 at 20°, and a titration curve could not be obtained. pK_1 was approximately 8·1.

The dissociation constants used in the calculations to be described are given in Table 2. It was assumed that NH_3^+ groups have a heat of ionization of +11000 cal. (Cohn & Edsall, 1943) and that the hydantoin ring structure has a heat of ionization similar to that of imidazole compounds, which is approximately +7000 cal. (Cohn & Edsall, 1943).

Reaction of diformylhomocystine

The pK values of the carboxyl groups of diformylhomocystine would be expected to be similar to those of dithiodipropionic acid and diformylcystine (i.e. less than about 5–5·5), so that kinetic experiments at pH 6·5 refer, within the experimental limits, to the completely ionized form.

Above pH 9, the reaction is reversible and all the sulphite is in the form of SO_3^{2-}. Rate constants (k_α) for the forward reactions between ionized diformylhomocystine and SO_3^{2-} ions at 0° and 25° were found from experiments in the pH range 9·2–11·2; at 0°, $k_\alpha = 0{\cdot}57 \pm 0{\cdot}04$ l. mole^{-1}/min.$^{-1}$ and at 25°, $k_\alpha = 3{\cdot}9 \pm 0{\cdot}2$ l. mole^{-1}/min.$^{-1}$. These figures are the means and standard deviations of at least sixteen determinations of k_α from a minimum of four kinetic experiments. Rate constants from experiments in borate and carbonate buffers were the same within experimental error.

The reaction is complex below pH 9, when the equilibrium $HSO_3^- \rightleftharpoons H^+ + SO_3^{2-}$ must also be considered, and apparent initial rate constants were estimated as before (Cecil & McPhee, 1955*b*). It was again found that the rate constant for reaction with HSO_3^- ions was negligible, and that the change in apparent rate constant with pH was described by the equation

$$k' = \frac{k_2 K_\phi}{K_\phi + [H^+]}, \qquad (1)$$

where k' is the apparent rate constant at a given hydrogen-ion concentration, k_2 is the rate constant for reaction with SO_3^{2-} ions (i.e. k_α above) and K_ϕ is the dissociation constant of HSO_3^-. Some results are given in Table 3, a mean value for pK_ϕ of 6·8 again being found from equation (1) from the experimental values of k' and k_2.

Table 3. *Reaction of diformylhomocystine with sodium sulphite at 25° and* pH *below 9*

	Extrapolated value for apparent rate constant	
pH	k' (l. mole^{-1} min.$^{-1}$)	pK_ϕ calc.
6·45	1·3	6·75
6·85	2·0	6·83
7·20	2·8	6·80

From measurements of the equilibrium concentrations of thiol in reaction mixtures at pH values below 9, the results in Table 4 were calculated as shown previously (Cecil & McPhee, 1955*b*). As with the three other thiol compounds studied, the SH group of *N*-formylhomocysteine has a heat of ionization of approximately +6000 cal./mole^{-1}.

Reactions of homocystine, homocystine hydantoin and cystine hydantoin

The kinetic expressions applying to these reactions, which are complicated by the presence in the disulphide molecules of groups whose state of ionization change in the pH range used, have been derived (Cecil & McPhee, 1955*b*). The rate constants for the reactions with SO_3^{2-} ions (k_2) of the zwitterion form of homocystine and the uncharged forms of homocystine hydantoin and cystine hydantoin were calculated from equation (1), by taking the value of K_ϕ ($1{\cdot}6 \times 10^{-7}$) found in experiments with other disulphides and using the apparent rate constants (k') measured in the pH range 4·8–7·0. The results are summarized in Table 5, and the mean values for k_2 are given in Table 9.

Above pH 9, the apparent rate constants (k'') of the forward reactions with SO_3^{2-} ions decrease as the pH is increased, the results for homocystine and homocystine hydantoin being shown in Table 6. (Kinetic experiments with cystine hydantoin could not be done at pH values above 9 owing to decomposition of the disulphide.) In both series of experiments, the results in borate were consistent with those in carbonate, as shown by the small range of values calculated for the forms of net charge −1

Table 4. *Results from equilibrium studies on the reaction of diformylhomocystine with sodium sulphite*

$$R.S.S.R + SO_3^{2-} \overset{K_1}{\rightleftharpoons} R.S^- + R.S.SO_3^-$$

Temp.	I	$10^2 K_1$	ΔH^0 (cal. mole^{-1})	ΔG^0 (cal. mole^{-1})	ΔS^0 (cal. mole^{-1} deg.$^{-1}$)
0°	0·09–0·11	0·41 ± 0·01	+7100	+2600	+15
25	0·08–0·11	1·23 ± 0·02	—	—	—

$$R.SH \rightleftharpoons R.S^- + H^+$$

		pK_{SH}			
0	0·09–0·11	10·37 ± 0·03	+5800	+13 600	−26
25	0·08–0·11	9·98 ± 0·02	—	—	—

Figures given in the third column are the means and standard deviations of at least six calculated values.

Table 5. *Reactions of homocystine, homocystine hydantoin and cystine hydantoin with sodium sulphite at* pH *below* 9

Substance	Temp.	pH	Extrapolated value for apparent rate constant, k' (l. mole^{-1} min.$^{-1}$)	Rate constants with SO_3^{2-}, k_2 (l. mole^{-1} min.$^{-1}$)
Homocystine	0°	4·85	0·2	18
	0	6·42	5·5	18
	0	6·63	6·5	16
	0	6·92	11	19
	25	4·88	2	167
	25	5·63	11	172
	25	5·98	20	152
	25	6·42	40	136
	25	6·63	60	150
Homocystine hydantoin	0	5·26	0·2	7
	0	5·84	0·7	7
	25	4·91	0·75	58
	25	5·38	2	54
	25	5·49	2·5	54
	25	6·22	12	57
Cystine hydantoin	0	5·30	1·3	42
	0	6·32	10	40
	25	4·79	3	304
	25	4·92	4	306
	25	5·51	14	285
	25	5·80	25	274

Table 6. *Reactions of homocystine and homocystine hydantoin with sodium sulphite at* pH *values above* 9

Substance	Temp.	pH	Apparent rate constant, k'' (l. mole^{-1} min.$^{-1}$)
Homocystine	0°	9·30	8·0
	0	9·96	2·4
	0	10·41	0·75
	0	11·12	0·45
	25	9·20	36·0
	25	9·70	14·6
	25	10·10	5·3
	25	11·10	2·3
Homocystine hydantoin	0	10·04	1·5
	0	10·80	0·86
	25	9·20	20·5
	25	9·83	9·1
	25	10·32	6·8
	25	11·07	5·0

Table 7. *Reactions of cystamine and homocystamine with sodium sulphite in acetate buffers at I 0·03*

Substance	Temp.	pH	Extrapolated value for apparent rate constant, k' (l. mole^{-1} min.$^{-1}$)	Rate constants with SO_3^{2-}, k_2 (l. mole^{-1} min.$^{-1}$)
Cystamine	0°	4·58	4	660
	0	4·75	5·5	620
	0	5·32	20	620
	25	4·56	40	6900
	25	4·58	40	6800
	25	4·68	50	6600
	25	5·39	250	6600
Homocystamine	0	4·76	2	220
	0	5·08	4	210
	0	5·40	9	230
	0	5·53	11	215
	25	4·52	14	2700
	25	4·62	17	2600
	25	5·05	45	2600
	25	5·21	60	2400

which are given in Table 9. (These were calculated from the experiments in Table 6.) The rate constants for the forms of net charge -2 which were found from experiments in carbonate buffers are also given in Table 9.

Experiments were not done at high enough pH values for the equilibrium constants for the reactions of homocystine and homocystine hydantoin (in forms of net charge -2) with SO_3^{2-} ions to be found.

Reactions of cystamine and homocystamine

The rate constants for the reactions of cystamine (net charge $+2$) and homocystamine (net charge $+2$) with SO_3^{2-} ions were found from experiments in acetate buffer (I 0·03) over the pH range 4·5–5·5, K_ϕ being taken to be $1{\cdot}6 \times 10^{-7}$ as above. The results are shown in Table 7, the mean k_2 values being given in Table 9.

The presence of buffer salts markedly affects the rates of these two reactions. Increasing the ionic strength lowers the rate constants for the reactions, and also phosphate, borate and carbonate inhibit the reaction. It has been found (Cecil & McPhee, 1955*b*) that rate constants for the cystine SO_3^{2-} reaction were lower in carbonate than borate, but a similar effect has not been observed with other compounds except the two now being discussed. The inhibitory effect of phosphate is shown by the fact that a value for k_2 of 3900 l. mole^{-1} min.$^{-1}$ was found at I 0·03 in phosphate, compared with 6700 l. mole^{-1} min.$^{-1}$ in acetate at the same ionic strength (Tables 7 and 8). The effect of ionic strength is illustrated by experiments in phosphate, when increasing the ionic strength from 0·03 to 0·05 lowered k_2 from 3900 to about 2400 l. mole^{-1} min.$^{-1}$ (Table 8); also by an experiment done in acetate, with the ionic strength brought to 0·10 by the addition of sulphate, k_2 then being 1800 l. mole^{-1} min.$^{-1}$. The k'' values found at pH values above 9 in borate and carbonate are too low for calculations of the rate constants for the various charged forms of cystamine to be made. Negative values for these rate constants were obtained even when a value of about 2000 l. mole^{-1} min.$^{-1}$ was used for the rate constant for the form of net charge $+2$. Experiments shown in Table 8 also indicate that, as with cystine, reaction is slower in carbonate than borate; for example, at pH 9·9, k'' is 100 l. mole^{-1} min.$^{-1}$ in borate, but is 77 l. mole^{-1} min.$^{-1}$ in carbonate.

Homocystamine showed the same general behaviour as cystamine.

Table 8. *Reaction of cystamine with sodium sulphite in various buffers at 25°*

pH	I	Extrapolated value for apparent rate constant, k' (l. mole^{-1} min.$^{-1}$)	Rate constants with SO_3^{2-}, k_2 (l. mole^{-1} min.$^{-1}$)
		Phosphate buffer	
6·17	0·05	450	2300
6·17	0·03	750	3900
6·46	0·05	800	2500
		Acetate with sulphate added	
5·32	0·10	57	1800
		Borate buffer Apparent rate constant k'' (l. mole^{-1} min.$^{-1}$)	
9·20	0·06	630	
9·55	0·09	180	
9·90	0·10	100	
		Carbonate buffer	
9·41	0·06	96	
9·91	0·07	77	
10·49	0·09	37	

DISCUSSION

Table 9 summarizes the kinetic data so far obtained on the reactions of disulphides with SO_3^{2-} ions, the influence of the net charge of the disulphide molecules on the rate constants being clearly demonstrated. Homocystine and its derivatives react more slowly than the corresponding cystine compounds, the largest differences being between the rate constants for the zwitterion forms of homocystine and cystine, which differ by a factor of about 7. However, the values of k_2 for all the homocystine derivatives are of the same order of magnitude as for the corresponding cystine derivatives, and it is clear that the most important factor governing the rates of reaction of disulphide bonds with SO_3^{2-} ions is the net charge in the immediate vicinity of the bonds. Total charge also influences the rate constants to a certain extent, uncharged cystine derivatives (diacetylcystine ester and the uncharged form of cystine hydantoin) reacting more closely than the zwitterion form of cystine; similarly, the uncharged form of homocystine hydantoin reacts more slowly than the zwitterion form of homocystine.

The work of Schöberl & Ludwig (1937) and Kassel & Brand (1938) is in qualitative agreement with that presented here, except for the reaction of cystamine with sulphite. They found that cystamine reacted more slowly than cystine, although Kassel & Brand (1938) observed that cystine ester did react more rapidly than cystine. Much higher ionic strengths were used in both these sets of experiments than in ours, which is a possible reason for the different results.

It is significant that the reactions of simple disulphides with potassium cyanide follow the same general pattern as the reactions with sulphite (Schöberl & Ludwig, 1937). The reacting species is almost certainly CN^- ions, and reactivity will be governed by charged groups in the disulphide molecules, although the variations in rate constants might be expected to be less than is the case with divalent SO_3^{2-} ions. Similarly, the reduction of disulphide bonds by thiol compounds involves, in alkaline solution at least, the attack of RS^- ions, so that variations in reactivity of disulphide bonds toward, say, alkaline thioglycollate (Walker, 1925) could be due to nearby charged groups. Coulomb forces will also influence the reactivity of disulphides toward OH^- ions (Tarbell & Harnish, 1951).

This work has been done at relatively low ionic strengths ($I < 0{\cdot}15$) where Coulomb forces exert their maximum effect. At high ionic strengths the

Table 9. *Summary of kinetic data*

Substance	Rate constants with SO_3^{2-}, k_2 (l. mole^{-1} min.$^{-1}$) (25°)	(0°)	Net charge on molecule	Activation energy, E_a (cal.)	Frequency factor, pZ_0, at 25° (l. mole^{-1} sec.$^{-1}$)	Entropy of activation, ΔS^* (cal. mole^{-1} deg.$^{-1}$)
Cystamine	6700†	630†	+2	15 300	$1{\cdot}6 \times 10^{13}$	+1
Diacetylcystine (ester)	265	37	0	12 800	1×10^{10}	−14
Cystine hydantoin	290	40	0	12 800	9×10^{9}	−14
Diformylcystine (ionized)	9	1·5	−2	11 500	4×10^{7}	−25
Dithiodipropionic acid (ionized)	1·1	0·13	−2	13 500	9×10^{7}	−24
Cystine‡						
2+,2 −	1100	140	0	13 300	9×10^{10}	−10
1+,2 −	(5–70)	(0·05–5)	−1	—	—	—
0+,2 −	3§	0·5§	−2	11 600	2×10^{7}	−27
Homocystamine	2600†	220†	+2	16 000	$1{\cdot}2 \times 10^{13}$	+1
Homocystine hydantoin	56	7	0	13 500	6×10^{10}	−10
	(12–15)	(1·5–2)	−1	—	—	—
	4·5§	0·7§	−2	12 000	4×10^{7}	−24
Diformylhomocystine (ionized)	3·9	0·57	−2	12 400	7×10^{7}	−24
Homocystine‡						
2+, 2 −	155	18	0	13 900	3×10^{10}	—
1+, 2 −	(20–30)	(1–1·5)	−1	—	—	—
0+, 2 −	2·0§	0·4§	−2	10 400	$1{\cdot}3 \times 10^{6}$	−32
Oxidized glutathione‡						
2+, 4 −	160	22	−2	12 800	6×10^{9}	−15
1+, 4 −	(40–50)	(2–4)	−3	—	—	—
0+, 4 −	10§	2§	−4	10 400	4×10^{8}	−29

† Values for k_2 in acetate buffers at I 0·03.
‡ Numbers of positive and negative charges/molecule in different states of ionization.
§ Values for k_2 in carbonate buffers.

differences in rate constants may not be so large, but the overall effects observed are so great that the differences would be expected still to exist.

Application to proteins. The variations in rates observed are large enough to show up as qualitative differences for disulphide bonds in proteins. For example, with a 20-fold molar excess of sulphite over disulphide at pH 7 at room temperature, the cystamine reaction is complete in less than 1 min., the cystine reaction is complete in 15 min., but the diformylcystine reaction takes over 30 hr. to reach completion. Thus under a standard set of conditions, protein disulphide bonds which had either no net charge or a net positive charge in their immediate environment would react readily with sulphite, whereas those associated with net negative charges would appear not to react. The pH range normally used in the study of proteins is about 4–6, where negative charges would be due to CO_2^- groups, but at higher pH values ionization of tyrosine OH groups would be expected to influence the rates of reaction of neighbouring disulphide bonds. Lindley & Phillips (1947) have suggested that the variations in reactivity of disulphide bonds in wool toward sulphite could be due to their environments, the reactive bonds being associated with carboxyl and acid amide side-chains, and the unreactive bonds having non-polar environments. However, the present work indicates that nearby carboxyl groups would make disulphide bonds unreactive toward sulphite, a non-polar environment allowing them to react readily unless steric hindrance were the limiting factor.

It is interesting that all the disulphide bonds of wool can react with oxidizing agents which are neutral molecules, but that only about 30 % of the total will react if the oxidizing agents are negative ions (Alexander, Hudson & Fox, 1950). Further, the rate of reaction with MnO_4^- ions decreases with increasing pH, so that it appears that charged protein groups play some part in these reactions.

When an ionic reactant is involved, it might be anticipated that Coulomb forces from nearby charged groups would greatly influence the rates of reaction of protein functional groups. However, the effect has rarely been considered in protein chemistry and the possible magnitude of such effects in typical reactions used for studying proteins does not appear to have been previously demonstrated. Similar studies on characteristic reactions of other amino acids and their simple derivatives would give information essential to an understanding of the chemistry of proteins. For example, 1-fluoro-2:4-dinitrobenzene reacts readily with both histidine and its methyl ester to form bisdinitrophenyl derivatives, but reaction is very slow with α-acetylhistidine and α-benzoylhistidine methyl ester (Sanger, 1945). Thus environment will influence the reactivity of protein imidazole rings toward FDNB, 1-fluoro-2:4-dinitrobenzene, and variations in reactivity have been observed (Porter, 1950).

Interactions between various protein functional groups (due to Coulomb forces, hydrogen bonding, etc.) would be expected to cause variations in the ionization constants of any one type of group (Laskowski & Scheraga, 1954), and there is evidence that the pK values of the tyrosine OH groups of native egg albumin may vary (Crammer & Neuberger, 1943). Rate constants for reactions involving different charged forms of the side chains would be considerably different. For example, the rate constant for the reaction of the OH form of tyrosine with hypoiodous acid is about 1000 times lower than that for the O^- form (Li, 1942). Similarly, the rates of oxidation of dithiol compounds depend on the pK values of the SH groups (Barron, Miller & Kalnitsky, 1947). Thus variation in the state of ionization of groups of a particular type at a given pH would be one of the factors leading to the apparent non-reactivity of protein functional groups under a given set of reaction conditions.

Configurational changes in protein structure, such as are involved in denaturation or in the stretching of fibres, will change the environments of many of the functional groups, thereby bringing about general differences in reactivity. For example, disulphide bonds which react slowly with SO_3^{2-} ions in a native protein (due to nearby CO_2^- groups) may react readily in the denatured state. (The converse is also true, so that, even though more disulphide bonds may be detected after denaturation, those found do not necessarily represent the total number in the protein.) Again, changes in pK values of groups on denaturation, as occurs with the tyrosine OH groups of egg albumin (Crammer & Neuberger, 1943), will cause a different reactivity if similar reaction conditions are used. It is always possible that groups may appear non-reactive after denaturation. For example, di*iso*propyl fluorophosphate (di*iso*propyl phosphorofluoridate) reacts with native chymotrypsin but with neither denatured enzyme nor with any of the constituent amino acids under the same conditions (Jansen, Nutting, Jang & Balls, 1950). It is likely that a study of the reaction of di*iso*propyl fluorophosphate with derivatives of certain amino acids would indicate which enzyme group is involved in this reaction, and also the reason for its apparent increased reactivity in the native protein.

SUMMARY

1. The methods of estimation and the kinetics of the reactions with sodium sulphite of the following disulphides are described: cystamine, homocyst-

amine, homocystine, cystine hydantoin, homocystine hydantoin, diformylhomocystine.

2. It has been found that positively charged compounds containing disulphide bonds react much faster with SO_3^{2-} ions than do disulphides with no net charge.

3. The pK of the thiol group of N-formylhomocysteine was calculated from equilibrium data. This group has a heat of ionization of approximately + 6000 calories.

4. The influence of chemical environment on the reactivity of protein functional groups is discussed.

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Changes in the Nucleic Acid Content of Ganglion Cells during Chromatolysis and after Stimulation

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This paper is concerned with the changes in total nucleic acid and deoxyribonucleic acid (DNA) in the superior cervical ganglion of the rabbit after section of the postganglionic fibres or stimulation of the preganglionic fibres.

In peripheral nerve it has been shown (Logan, Mannell & Rossiter, 1952*b*) that after section of the nerve the total nucleic acid increases in the distal stump to a maximum at about the sixteenth day after section and that this rise in total nucleic acid is due largely to a marked increase in the ribonucleic acid (RNA) fraction. Similar, but less marked, changes can be detected during the first 48–96 hr. after nerve section, and furthermore it has been shown that intense stimulation of the normal nerve trunk produces a reduction in the DNA fraction while leaving the total nucleic acid unchanged (Causey & Stratmann, 1956). Hydén (1943), using Caspersson's ultraviolet-absorption technique, showed a decrease in the RNA content of the anterior-horn cells of the spinal cord after intense motor activity.

It was apparent that if the more simple chemical method of nucleic acid estimation was to be applied to nerve-cell bodies, a more uniform and discrete group of cells than those used by Hydén would have to be employed. The cells of the superior cervical ganglion were used. The proportion of nerve cells to supporting tissues and fibres is high, and it is possible to subject the cells both to section of the nerve processes by cutting the postganglionic fibres and to excitation by stimulation of the preganglionic trunk.

EXPERIMENTAL

Rabbits of about 2·5 kg. body weight were used throughout; no selection was made for breed or sex. All survival operations were carried out under Nembutal (pentobarbitone sodium, B.P.) and ether anaesthesia. The specimens were dissected out under urethane anaesthesia. The superior

cervical ganglia were dissected out carefully, freed of fat and fibrous connective tissue, and weighed wet. After section of the postganglionic fibres there is a considerable increase in the wet weight of the ganglion; therefore the wet weight of the normal ganglion of the opposite side was used as a measure of the original weight of the ganglion subjected to operation, and the results have been calculated in these terms. A similar procedure was also used for the stimulated ganglia which had been in contact with liquid paraffin. To estimate the error of this procedure a number of ganglia from opposite sides of normal rabbits were weighed, and although individual pairs varied considerably in weight the differences in the wet weight between pairs of ganglia amounted to only $0{\cdot}24 \pm 0{\cdot}048$ mg. for fourteen pairs of ganglia. The average wet weight of a ganglion was about 10 mg.

Chromatolysis of the nerve cells was produced by section of all the cephalic branches of the ganglion close to the body of the ganglion. The animals were killed 7 or 14 days later. Excitation of the normal ganglion was produced by stimulation of the cervical sympathetic chain at a frequency of 300 cyc./sec. for a period of 3 hr., at a stimulus strength just supramaximal as judged by the compound action potential observed on an oscilloscope. The nerve and stimulating electrodes were immersed in liquid paraffin.

The ganglia were analysed for total nucleic acid and DNA. The method of analysis was essentially similar to that described by Logan, Mannell & Rossiter (1952*a*). The ganglia, cleaned of any adherent fatty and connective tissue, were weighed wet and homogenized in ice-cold 10% (w/v) trichloroacetic acid in a test-tube homogenizer. Acid-soluble P and lipid were removed from the homogenate by treatment with further quantities of trichloroacetic acid, ethanol and ether–ethanol according to the method of Schneider (1945). One series of samples was then extracted for 15 min. at 90° with 5% (w/v) trichloroacetic acid. This was used for determination of total nucleic acid by ultraviolet absorption. The other series of samples, after treatment as above for removal of acid-soluble P and lipid, was hydrolysed for 18 hr. at 37° in N-KOH. DNA was precipitated by the addition of 5N-HCl and 5% (w/v) trichloroacetic acid to the hydrolysate. The DNA was removed by centrifuging. The precipitated DNA was then extracted with 5% (w/v) trichloroacetic acid for 15 min. at 90° and estimated by ultraviolet absorption.

The final estimation of total nucleic acid and DNA was made by determination of the optical densities of the solutions at a wavelength of 268·5 mμ. with a Hilger Uvispek spectrophotometer.

Trichloroacetic acid itself absorbs appreciably at this wavelength, therefore a blank consisting of 5% (w/v) trichloroacetic acid was used. As trichloroacetic acid tends to be broken down during heating at 90°, it was necessary for the samples and blanks to be heated for exactly the same length of time. The optical density of 5% (w/v) trichloroacetic acid after heating for 15 min. at 90° was found to be approximately 0·150 at 268·5 mμ. in a 1 cm. cell.

The method was standardized by the use of stock solutions of DNA and RNA obtained commercially from Harrington Bros. The results are expressed in terms of mg. of nucleic acid P/100 g. wet wt. of tissue. The standard curves relating absorption and mg. of nucleic acid P were constructed by use of the stock solutions. The absorption characteristics and P content of these standards are shown in Table 1.

Since the amount of superior cervial ganglion tissue available was small it was not possible to estimate both total nucleic acid and DNA on the one sample from a single animal. Hence two series of experiments were carried out, in one of which total nucleic acid was estimated, and in the other DNA. Therefore a direct calculation of the RNA content (total nucleic acid — DNA) in each case, as was done previously (Logan *et al.* 1952*a*, *b*; Causey & Stratmann, 1956), was not possible.

A number of estimations of the water content of the tibial nerves of adult and immature rabbits has been carried out by weighing pieces of nerve before and after heating to constant weight in an oven at 110°.

RESULTS

The results are summarized in Tables 2–5. In the normal superior cervical ganglion (Table 2*a*) there is about 44 mg. of nucleic acid P/100 g. wet wt. of tissue, and of this 13 mg. is represented by the DNA fraction. Seven days after section of the postganglionic fibres (Table 2*b*) total nucleic acid was found to have doubled, and by the 14th day to have reached three times the original amount in the ganglion (130 mg.). At the same time the DNA fraction had also risen, so that at both the 7th and 14th day after section the DNA was about four times that originally present (50 mg.). Whilst direct calculation of the difference, total nucleic acid minus DNA, is impossible for the reasons given above, the difference between the means of the two groups of estimations appears to rise very sharply up to about 80 mg. of nucleic acid P/100 g. wet wt. of tissue by the 14th day. The fraction concerned in this rise would be, presumably, RNA.

Table 1. *Phosphorus content and absorption characteristics of standard preparations of DNA and RNA*

ϵ (P), the atomic extinction coefficient with respect to P, is defined: $\epsilon(\mathrm{P}) = \frac{30{\cdot}98E}{cl}$, where $E = \log I_0/I$, c = concentration of P in g./l., and l = thickness of absorbing solution in cm. (Chargaff & Zamenhof, 1948. TCA: trichloroacetic acid.)

	λ (mμ.)	ϵ (P)	P content (%)
DNA			
In water	259 ($\lambda_{max.}$)	8980	6·9
After heating for 15 min. in 5% TCA	268·5	9820	—
RNA			
In water	258 ($\lambda_{max.}$)	9450	8·5
After heating for 15 min. in 5% TCA	268·5	9820	—

Table 2. *Nucleic acid content of the superior cervical ganglion of the rabbit*

(*a*) From normal rabbits, (*b*) from rabbits in which all the cephalic branches of the ganglion were sectioned 7 days prior to biopsy, and (*c*) from rabbits in which all the cephalic branches of the ganglion were sectioned 14 days prior to biopsy. For the normals the results are expressed in mg. of nucleic acid P/100 g. wet wt. of ganglion. For operated ganglia results are expressed in terms of mg. of nucleic acid P/100 g. wet wt. of the unoperated ganglion from the opposite side. Number of estimations is given in parentheses after each result. Results are given as mean ± standard error of mean.

	Total nucleic acid	DNA
a	43·7±2·20 (29)	13·0±0·60 (39)
b	90·4±6·47 (8)	48·5±5·54 (10)
c	128·7±3·84 (9)	51·0±2·22 (9)

Table 3. *Nucleic acid content of the superior cervical ganglion of the rabbit*

Ganglia were (*a*) excited by stimulating the cervical sympathetic chain in the neck for 3 hr. at a frequency of 300 cyc./sec. under liquid paraffin, and (*b*) soaked in paraffin for a similar period and under exactly the same conditions but without stimulation. The results are expressed in terms of mg. of nucleic acid P/100 g. wet wt. of ganglion taken from the untreated side. Number of estimations is given in parentheses after each result.

	Total nucleic acid	DNA
a	43·3±1·95 (10)	32·8±3·65 (9)
b	—	11·8±0·99 (9)

Table 4. *Mean nucleic acid content and its standard error of* (*a*) *the superior cervical ganglion of rabbits* 1–2 *days old,* (*b*) *the tibial nerve of rabbits* 1–2 *days old and* (*c*) *the connective-tissue sheath of the tibial nerve of adult rabbits*

Results are expressed in terms of mg. of nucleic acid P/100 g. wet wt. of fresh tissue. Number of estimations is given in parentheses after each result.

	Total nucleic acid	DNA
a	81·4±2·67 (7)	18·6±2·40 (5)
b	49·2±3·46 (14)	17·6±1·17 (16)
c	17·1±1·70 (7)	8·2±0·98 (6)

Table 5. *Mean water content and its standard error of* (*a*) *the tibial nerve of adult rabbits and* (*b*) *the tibial nerve of rabbits* 1–2 *days old*

Results are expressed as the percentage of water in the wet nerve. Number of estimations is given in parentheses after each result.

	Water content (%)
a	67·8±1·64 (8)
b	75·4±1·17 (8)

When the preganglionic fibres to the ganglion were subjected to a long period of fast repetitive stimulation (Table 3*a*) no change was found in the total nucleic acid content of the ganglion, but a sharp rise in the DNA was observed, the content increasing nearly three times to some 33 mg. of DNA P/100 g. wet wt. of tissue. Control estimations (Table 3*b*) were made on ganglia left *in situ* and surrounded with liquid paraffin for 3 hr. without stimulation. There was no significant difference in the DNA content of these ganglia from the normals.

In an attempt to assess the significance of these marked changes that occur in total nucleic acid and DNA on degeneration and stimulation, estimations of the total nucleic acid and DNA content were made in (1) very immature rabbits (1–2 days old), and (2) the fibrous-tissue sheath of the tibial nerve of adult rabbits. The results of these estimations are shown in Table 4. With immature rabbits, at least four superior cervical ganglia had to be pooled in order to obtain enough tissue for analysis. The tibial nerves, however, were of sufficient size to permit analysis to be carried out on each nerve.

It will be seen that both the superior cervical ganglia and the tibial nerves of immature rabbits show high values of both total nucleic acid and DNA as compared with the corresponding tissues of the adult rabbit. The superior cervical ganglia have a total nucleic acid content twice that found in the adult, although the DNA was not much higher. The tibial nerves show both total nucleic acid and DNA levels approximately seven times the adult levels (Causey & Stratmann, 1956).

The fibrous-tissue sheath estimations were carried out on epineural sheath stripped from adult tibial nerves. The results of these estimations are also shown in Table 4. These estimations show high levels of both total nucleic acid and DNA, of the same order of magnitude as that found in adult whole tibial nerve.

The results of estimations of water content of the tibial nerves of adult and immature rabbits are shown in Table 5. It will be seen that there is very little difference in water content between the two groups of nerves, the tibial nerves of young rabbits (1–2 days old) having, as would be expected, a slightly higher water content than adult nerve.

DISCUSSION

The great changes in the nucleic acids of the superior cervical ganglion reported in this paper provide further evidence of the lability of these components in nervous tissue (Causey & Stratmann, 1956). The nucleic acid content, expressed in terms of wet weight of tissue, in superior cervical ganglia and nerve of young rabbits (1–2 days old), has been found to be much greater than that found in adults.

This difference cannot be due to differences in water content between adult and immature tissue, as is shown in Table 5. Nor does it seem likely that, in tibial nerves, differences in amount of epineural sheath could be responsible, for it has been found that sheath taken from adult nerves contains considerable quantities of nucleic acids. It seems most likely that this higher level of nucleic acid, particularly in the RNA fraction, is associated with growth processes in the young animal.

Total nucleic acid is known to increase in the peripheral stump during degeneration of nerve and closely follows proliferation of Schwann cells (Logan *et al.* 1952*b*), but it is uncertain how far or what proportion of the increase during the first 48–96 hr. is due to changes in Schwann cells (Causey & Stratmann, 1956). The increase of nucleic acid content in the ganglia after severance of the axons, on the other hand, is likely to be due mainly to changes in the nerve-cell bodies, and is, presumably, linked with the process known to histologists as chromatolysis (Gersh & Bodian, 1943; Hydén, 1943). The changes reported here show at first an increase involving both DNA and RNA, and later mainly RNA, perhaps in association with the synthesis of new axoplasm.

The changes in nucleic acids as a result of stimulation are more difficult to evaluate. In a peripheral nerve trunk, after stimulation, the total nucleic acid content remains constant but RNA was found to increase while DNA falls (Causey & Stratmann, 1956). However, preganglionic excitation of the superior cervical ganglion leads to an increase in DNA and a fall in RNA without change in the total nucleic acid content of the cells. The cellular processes involved in these changes remain obscure, but it is clear that the nucleic acids of both cell body and nerve fibre play an intimate part in functional activities. The opposing trends in the ganglion and the peripheral nerve trunk could perhaps be attributed to the necessity of re-establishing a steady state.

SUMMARY

1. Total nucleic acid and deoxyribonucleic acid (DNA) have been determined in the superior cervical ganglion of the rabbit after section of the post-ganglionic fibres and after stimulation of the pre-ganglionic fibres.

2. The normal levels of total nucleic acid and DNA in the superior cervical ganglion were 44 mg. of nucleic acid P/100 g. wet wt. of tissue and 13 mg. of nucleic acid P/100 g. wet wt. of tissue, respectively.

3. Total nucleic acid and DNA both increased after section of the postganglionic fibres. At 14 days after section the level of total nucleic acid was 130 mg. and of DNA 50 mg. The main increase is presumably in the ribonucleic acid fraction.

4. After stimulation of the preganglionic fibres total nucleic acid remained unchanged but DNA increased markedly.

5. Estimations of total nucleic acid and DNA content of the superior cervical ganglion and tibial nerve of very immature rabbits are also reported.

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Hormonal Influences on the Incorporation of Injected Precursors into the Protein and Ribonucleic Acid of Liver Cytoplasm

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When differential centrifuging was performed on rat-liver homogenates, prepared in 0·25 M sucrose solution, evidence was obtained that certain cytoplasmic elements are selectively affected by changes in the hormonal status of the animal (Reid, 1955, 1956). For example, it appeared that the mitochondrial fraction is the site of the fall in liver ribonucleic acid (RNA) which is known to occur in untreated hypophysectomized rats and which, as Geschwind, Li & Evans (1950) have suggested, may reflect a reduction in protein synthesis.

Lee & Williams (1953) gave growth hormone to intact rats and observed an enhancement of the incorporation of labelled cystine, given by injection, into the protein of nuclear and mitochondrial fractions from liver. Otherwise there is no published evidence indicative of a relationship between hormonal status and the rate of protein synthesis, or of RNA synthesis, in the fractions obtained from liver by differential centrifuging.

Untreated hypophysectomized rats, hypophysectomized rats treated with growth hormone, and untreated adrenalectomized rats have now been compared with intact rats with respect to the incorporation, *in vivo*, of [1-^{14}C]leucine into the protein of liver cytoplasmic fractions, and of [6-^{14}C]-orotic acid into the RNA of these fractions. Some of the observations have been briefly reported elsewhere (Reid & Stevens, 1956).

EXPERIMENTAL

Isotopically labelled compounds. DL-[1-^{14}C]Leucine was obtained from the Radiochemical Centre, Amersham.

[6-^{14}C]Orotic acid was prepared by the method of Heidelberger & Hurlbert (1950) from ethyl diethoxyacetate (Johnson & Cretcher, 1915) and ethyl [1-^{14}C]acetate (Sakami, Evans & Gurin, 1947). Three millimoles (3·3 mc) of sodium [1-^{14}C]acetate gave 253 mg. of recrystallized orotic acid monohydrate; equivalent weight 176 (theory 174); decomposition point 345°; yield from $Ba^{14}CO_3$, 45%. The specific activity was 6·4 μc/mg.

Animals and injections. The rats were albino males of the Institute colony, of age 10 weeks and body weight about 220 g. at the time of autopsy. Operations were performed at 7–8 weeks of age under Avertin (tribromoethanol) anaesthesia. The criterion for completeness of hypophysectomy, which was performed by the usual parapharyngeal approach, was diminution of testicular and adrenal weights to below one-third of those for equivalent intact animals. Bilateral adrenalectomy was performed by the usual dorsal approach; the rats were given 1% NaCl solution in place of drinking water, and were examined at autopsy for the possible presence of nodules of adrenal tissue.

The operated rats, together with intact control rats, were kept in individual cages on wire grids, and were maintained on the '20% protein diet' described by Elson (1952); the food intake of the intact and adrenalectomized rats was restricted to that of the hypophysectomized rats (usually 10 g./day, admixed with water). For 8 days before autopsy, some of the hypophysectomized rats received subcutaneous injections of growth hormone (Armour, Lot 527093B; thyrotrophin content less than 0·06 U.S.P. unit/mg.) at a dose level of 0·3 mg./day; these rats showed an average weight gain of 2·5 g./day. The average changes in weight for untreated hypophysectomized rats, for adrenalectomized rats and for intact rats were −1·0, +1·2 and +1·0 g./day respectively.

The rats were arranged in groups of four, with one or two intact control rats in each group. The rats in each group were killed on the same day, and the liver homogenates centrifuged simultaneously. To minimize the effect of any variations in environment or technique, the data for experimental rats were not directly averaged over all the groups, but were compared with those for corresponding control rats and the differences averaged. Altogether five untreated hypophysectomized rats, five growth-hormone-treated hypophysectomized rats and seven adrenalectomized rats were used in the study of leucine incorporation. The corresponding numbers for the study of orotic acid incorporation were four, four and six.

The isotopically labelled compounds were given by intraperitoneal injection usually in a volume of 0·4 ml. at about pH 8·5. The dose of DL-[1-^{14}C]leucine was 2·05 mg., equivalent to 20 μc. The rats were killed 90 min. after the injection, at which time the radioactivity of the liver protein had barely attained a 'plateau' in intact rats. The dose of [6-^{14}C]orotic acid was 1·25 mg., equivalent to 8 μc. The rats were killed 135 min. after the injection; at this time, as also found by Hurlbert & Potter (1952), the radioactivity of the liver RNA had not attained a plateau even in the nuclear fraction, the RNA of which shows the most rapid incorporation. Each rat received the same dose of leucine or orotic

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acid irrespective of body weight, the dose being below the saturation level (cf. Keller, Zamecnik & Loftfield, 1954); the data of Table 1 include appropriate corrections for differences in body weight.

Isolation of tissue fractions. Each rat was finally anaesthetized with Nembutal (pentobarbital sodium), after an overnight fast. The liver was perfused *in situ* with cold 0·25M sucrose solution, after (in some experiments) withdrawal of a blood sample from the heart. The radioactivity of the liver protein was no higher if perfusion was omitted, in contrast with the findings of Lee, Anderson, Miller & Williams (1951).

The liver was quickly excised and weighed. A weighed portion was homogenized in cold 0·25M sucrose solution (12 ml./4 g. of liver) in a chilled homogenizer, with a Teflon pestle, as supplied by Arthur Thomas and Co., Philadelphia. The homogenates from the rats in each group were centrifuged for 10 min. at 600 *g* (3500 rev./min.). The debris thus obtained was rehomogenized in sucrose solution as before, and recentrifuged. These and subsequent centrifugings were performed at 0–3° in the high-speed head (Cat. no. 69404) of a refrigerated Medium centrifuge (Measuring and Scientific Equipment Ltd.); supernatant fluids were in general removed by pipette.

In the study of orotic acid incorporation, the debris (nuclear fraction) from the second centrifuging was resuspended in sucrose, and a portion set aside for treatment with trichloroacetic acid solution as described below. In the study of leucine incorporation the nuclear fraction was discarded, since the radioactivity of contaminating cytoplasmic protein would have masked any change in that of nuclear protein.

The supernatant fluids from the two centrifugings were combined to give the cytoplasmic fraction, a portion of which was set aside for treatment with trichloroacetic acid solution. The remainder was centrifuged for 20 min. at 10 000 *g* (10 500 rev./min.). The material thus sedimented was separated into an underlying mitochondrial fraction and a fluffy layer, the latter being dislodged with 2 ml. of 0·25M-sucrose solution by a swirling motion and removed by decantation. Because of the nature of this technique and of slight day-to-day variations in centrifuging speed, the yield of fluffy layer was rather variable.

The supernatant fluid was combined with the washings obtained on separately washing and recentrifuging the mitochondrial fraction and the fluffy layer. Further centrifuging, for 90 min. at 14 500 *g* (13 000 rev./min.), gave a microsomal fraction and a supernatant fraction. In some experiments a portion of the latter fraction was further centrifuged in a Spinco preparative ultracentrifuge, for 60 min. at 145 000 *g* (40 000 rev./min., Rotor 40), to sediment an ultracentrifugal fraction.

The various fractions thus obtained (including any blood samples) were treated with cold trichloroacetic acid solution (to 7%, w/v) and centrifuged after 15 min. The insoluble material was subjected to two thorough washings with cold 7% trichloroacetic acid solution, followed by treatment with hot ethanol and with hot ethanol–chloroform mixture (3:1, v/v). The products were finally dried overnight at 80°, weighed, and ground to a fine powder in a mortar.

The data for heart, kidneys and lungs were obtained with tissue which had been stored in the frozen state, and finally treated with trichloroacetic acid solution and lipid solvents as above.

Analyses. For the determination of radioactivity, each powdered sample was packed into the well (1 sq.cm.) of a polyethylene planchet, and counted in a thin-window counter. Duplicate counts agreed within 5%, the number of counts/min. being seldom less than five times background. Counting was performed at infinite thickness, except in the few cases where less than 30 mg. of material was available; in those cases an appropriate correction was applied.

L-Leucine and orotic acid are known to follow quite specific anabolic pathways, leading to protein and to nucleic acid respectively (Keller *et al.* 1954; Hurlbert & Potter, 1952). In the study of leucine incorporation, it was verified that the radioactivity of cytoplasmic fractions was undiminished after treatment with hot trichloroacetic acid solution (5%, 15 min. at 90°) to remove RNA, or with ninhydrin at pH 4·7 in the presence of unlabelled leucine as in the experiments of Winnick (1950). In the study of orotic acid incorporation, it was shown that cytoplasmic fractions had negligible radioactivity after treatment with hot trichloroacetic acid solution; moreover, when nuclear fractions were freed from RNA by the procedure of Schmidt & Thannhauser (1945), the DNA-containing residues gave very low counts which showed no consistent dependence on the hormonal status of the animal.

The RNA content of defatted fractions was measured by determining the extinction, at 260 mμ., of acidified alkaline digests (cf. Schmidt & Thannhauser, 1945; Keller *et al.* 1954).

RESULTS

Values for radioactivity can usefully be expressed as percentage recovery, in protein or RNA, of injected isotope. As is evident from the upper part of Fig. 1, the amount of injected leucine recovered in the cytoplasmic protein of liver was diminished after hypophysectomy, especially if growth hormone had been given, and was also somewhat diminished after adrenalectomy. The several fractions isolated from cytoplasm by differential centrifuging shared proportionately in these changes. The difference between untreated and growth-hormone-treated hypophysectomized rats was almost significant ($P<10\%$) in the cytoplasmic and microsomal fractions.

As is shown in the lower part of Fig. 1, the amount of orotic acid recovered in the crude nuclear material was rather variable; that in the cytoplasm as a whole tended to be low after hypophysectomy but was markedly raised after adrenalectomy. After hypophysectomy, the amounts incorporated into the RNA of the mitochondrial fraction, the fluffy layer and the microsomal fraction were decreased, in proportion to the amounts incorporated into these fractions in the intact controls. The supernatant and ultracentrifugal fractions did not, however, show a proportionate decrease. There was no indication of a difference in the incorporation of orotic acid between untreated and growth-hormone-treated hypophysectomized rats. Adrenalectomized rats had a diminished incorporation of

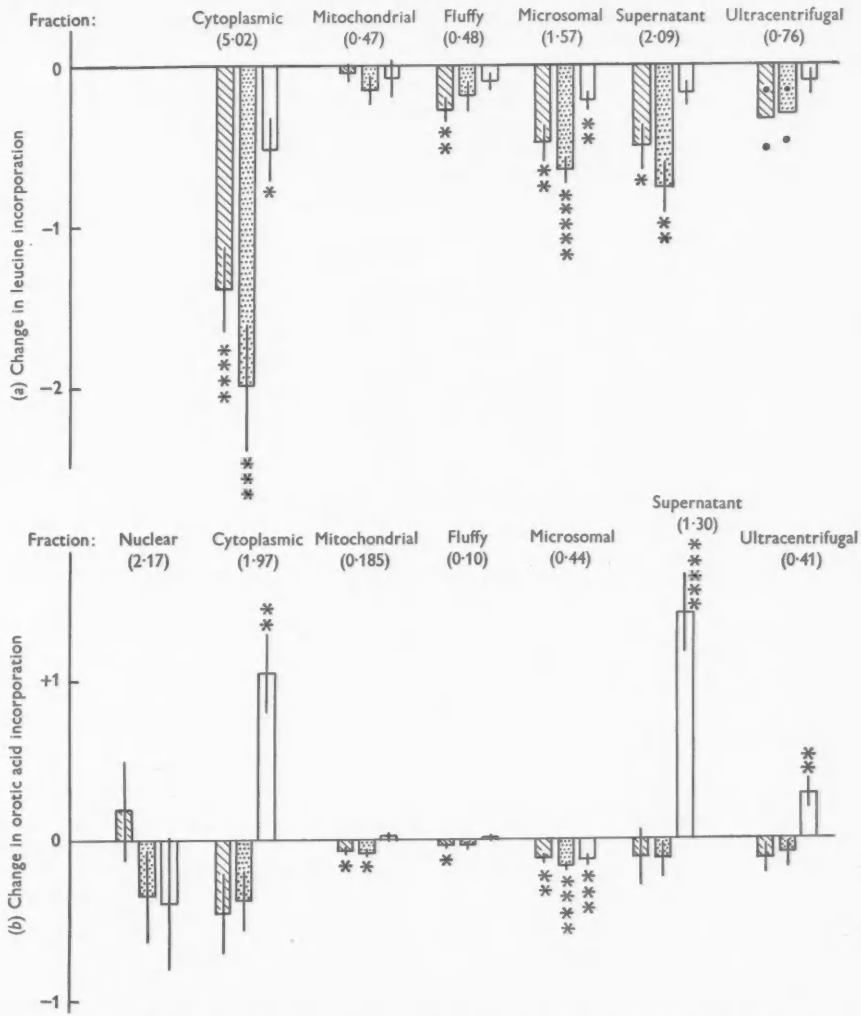

Fig. 1. Comparison of untreated hypophysectomized rats (bars with diagonal lines), of growth-hormone-treated hypophysectomized rats (bars with stipple), and of adrenalectomized rats (open bars) with intact rats, with respect to: (*a*) incorporation of DL-[1-^{14}C]leucine into liver protein, as percentage recovery of injected isotope; (*b*) incorporation of [6-^{14}C]orotic acid into liver RNA, as percentage recovery of injected isotope. The figures in parentheses are the mean values (percentage recovery) found for the intact rats. Vertical lines represent standard errors; where only two values were obtained they are indicated by large dots. Significant differences are denoted * ($P<5\%$), ** ($P<2{\cdot}5\%$), *** ($P<1\%$), **** ($P<0{\cdot}5\%$) or ***** ($P<0{\cdot}1\%$). The number of degrees of freedom (D.F.) is as given in Table 1, with the following exceptions for the orotic acid series; nuclear fraction, D.F. = 4 (hypophysectomized rats, either untreated or treated with growth hormone) or 6 (adrenalectomized rats); cytoplasmic fraction, D.F. = 4 (for each group); other fractions, D.F. = 7 (adrenalectomized rats only).

Table 1. *Specific radioactivity values for blood proteins and for fractions isolated from livers*

Animals were killed 90 min. or 135 min. after injection of radioactive leucine or orotic acid respectively. The standard error is given after each mean difference, except where less than four values were obtained. The number of degrees of freedom is given parenthetically. *P* denotes the probability that the difference could be due to chance.

Fraction	Control rats: mean value	Experimental rats: difference from corresponding control rats		
		Hypophysectomized	Hypophysectomized + growth hormone	Adrenalectomized
Incorporated leucine: μc/g. of defatted protein fraction, after injection of 10 μc of DL-leucine/100 g. body wt.				
Blood	0·46	−0·06±0·053 (4)	−0·07±0·043 (4)	−0·01±0·019 (3)
Cytoplasmic (total)	1·45	−0·46±0·135 (4; $P<5\%$)	−0·50±0·113 (4; $P<2·5\%$)	−0·30±0·115 (7; $P<5\%$)
Mitochondrial (without fluffy layer)	1·04	−0·29±0·044 (4; $P<0·5\%$)	−0·41±0·050 (4; $P<0·5\%$)	−0·18±0·071 (6; $P<5\%$)
Fluffy layer	1·41	−0·37±0·145 (3)	−0·17±0·165 (4)	−0·02±0·065 (6)
Microsomal	1·90	−0·43±0·153 (4; $P<5\%$)	−0·52±0·083 (4; $P<0·5\%$)	−0·02±0·060 (6)
Supernatant	1·23	−0·42±0·126 (4; $P<5\%$)	−0·46±0·111 (4; $P<2·5\%$)	−0·17±0·052 (6; $P<2·5\%$)
Ultracentrifugal (from supernatant)	1·72	−0·58 (1)	−0·62 (1)	−0·10±0·098 (3)
Incorporated orotic acid: μc/g. of RNA, after injection of 4 μc of orotic acid/100 g. body wt.				
Nuclear	13·0	+0·25 (2)	−0·95 (2)	−3·70 (2)
Mitochondrial (without fluffy layer)	2·16	−0·03±0·250 (4)	−0·62±0·308 (4)	+0·78±0·322 (6)
Fluffy layer	2·39	−0·54±0·320 (4)	−0·48±0·207 (4)	−0·17±0·416 (6)
Microsomal	2·44	−0·70±0·306 (4)	−0·92±0·384 (4)	−0·88±0·375 (6)
Supernatant	6·15	−0·51±0·560 (4)	−0·89±0·834 (4)	+1·73±0·45 (6; $P<1\%$)
Ultracentrifugal from supernatant)	4·28	−0·92±0·588 (4)	−1·05±0·255 (4; $P<2·5\%$)	+0·39±0·238 (4)

orotic acid into the microsomal fraction, but an increased incorporation into the supernatant fraction: this increase was not mainly located in the ultracentrifugal fraction.

In Table 1, the data are expressed in terms of specific activity rather than of recovery of injected isotope. The rough assumption may be made that the total leucine in the protein of the defatted fractions represents a constant percentage of the weight of the fractions.

It will be noted that the concentration of labelled leucine in the whole protein of blood was not significantly changed by hypophysectomy or by adrenalectomy, but that the concentration in the liver fractions showed the same trends as were observed with the values for percentage recovery. The specific activity values (not determined in every instance) for the RNA of liver fractions from rats given orotic acid also showed changes roughly parallel to those in percentage recovery; in particular it will be noted that the increased recovery of isotope in the supernatant fraction after adrenalectomy was accompanied by a significant, although relatively smaller, rise in specific activity.

With tissues other than liver, the recovery of isotope was rather variable, probably because of the short interval between injection of isotope and autopsy (cf. Lee *et al.* 1951). Adrenalectomy did not significantly change the incorporation of leucine into the protein of heart, kidney or lung. There were no significant hormonal effects on the incorporation of orotic acid by the kidney or lung.

DISCUSSION

The finding that the incorporation of leucine into the protein of the various cytoplasmic elements from liver is diminished after adrenalectomy, and especially after hypophysectomy, is in agreement with observations made with whole liver by Lee & Williams (1952) and by Clark (1953). The observation that the incorporation of orotic acid into the

RNA of cytoplasmic elements is diminished after hypophysectomy is also in agreement with previous reports (Fraenkel-Conrat & Li, 1949; Logan, Heagy & Rossiter, 1955). The divergent effects of adrenalectomy on the supernatant fraction and on the microsomal (and possibly the nuclear) fraction, with respect to the incorporation of orotic acid, might account for the failure of Logan *et al.* (1955) to demonstrate any effect of adrenalectomy on the incorporation of labelled phosphate into the RNA of liver as a whole.

In contrast with the present findings, administration of growth hormone to hypophysectomized rats has been reported to cause an increased incorporation of labelled amino acids into liver protein (Lee & Williams, 1952; Gaebler, 1955), and also a 'suggestive increased turnover' of a crude nucleic acid fraction from liver after injection of labelled phosphate (Fraenkel-Conrat & Li, 1949). However, this nucleic acid fraction was compared with plasma inorganic phosphate, the specific activity of which is decreased when growth hormone is given (Geschwind, Li & Evans, 1951). Moreover, it is conceivable that the apparent effects of growth hormone in the experiments cited were due to increased food consumption. Preliminary experiments in this laboratory have indicated that when intact rats are given food *ad lib.*, rather than in restricted amount, as in the present study, greater amounts of leucine and of orotic acid are incorporated. The suggestion of Bartlett & Glynn (1950), that there is actually a translocation of protein from liver to muscle in hypophysectomized rats kept on a restricted food intake and given growth hormone, is compatible with the present findings.

Changes in incorporation, as now observed, can be regarded as indicative of changes in the rate of synthesis of protein or RNA only if neither the size of the liver pool, with respect to the immediate precursors of protein and of RNA, nor the extent of labelling of the pool, is markedly changed. There is published evidence (Awapara, Marvin & Wells, 1949; Kit & Awapara, 1954) to suggest that these conditions were probably satisfied in the study of leucine incorporation after adrenalectomy. There is indirect evidence, from experiments with labelled phosphate (Geschwind *et al.* 1951), that the changes in incorporation after hypophysectomy probably do not merely reflect changes in the pool. Since, however, the pool was not directly studied in the present experiments and since measurements were made after only a single interval of time, any interpretation of the hormonal effects in terms of changes in rates of synthesis must be regarded as purely tentative.

The data of Fig. 1 are compatible with the view that RNA, particularly that in the microsomal fraction, plays a role in protein synthesis (cf. Keller *et al.* 1954; Brachet, 1955). This fraction showed a reduced incorporation both of leucine and of orotic acid after hypophysectomy or adrenalectomy. With the mitochondrial fraction and the fluffy layer the data give some indication of such a parallelism, but little elucidation of the previous observation (Reid, 1955, 1956) that the amount of RNA, in these fractions analysed conjointly, falls after hypophysectomy and is restored by growth hormone.

In the case of the supernatant fraction, however, hypophysectomy lowered the incorporation of leucine without proportionately lowering that of orotic acid. Moreover, adrenalectomy markedly increased the incorporation of orotic acid into the supernatant and ultracentrifugal fractions, without increasing the incorporation of leucine. A possible interpretation of these data is that the ultracentrifugal fraction, and particularly the whole supernatant fraction, may contain some RNA which has no connexion with protein synthesis. It should be pointed out that microsomal fractions as prepared in some studies of amino acid incorporation (Hultin, 1955; Littlefield, Keller, Gross & Zamecnik, 1955; Simkin, 1955) would comprise both the microsomal fraction and the ultracentrifugal fraction as now prepared.

In the absence of data for the precursor pool, it is of course uncertain whether the actual rate of synthesis of the RNA in the supernatant fraction rose after adrenalectomy, particularly since this RNA might be derived in part from the nucleus (cf. Smellie, McIndoe, Logan, Davidson & Dawson, 1953) or might itself be a precursor of microsomal RNA (Barnum, Huseby & Vermund, 1953). Evidently, however, it is unlikely that the effect of adrenalectomy in increasing the amount of RNA in the supernatant fraction (Reid, 1955, 1956) is attributable merely to an enhanced loss of RNA from cytoplasmic particles during their isolation.

SUMMARY

1. Untreated hypophysectomized rats, hypophysectomized rats treated with growth hormone and adrenalectomized rats have been compared with intact rats, kept on the same food intake, with respect to the incorporation of DL-[1-^{14}C]leucine into liver protein and of [6-^{14}C]orotic acid into liver ribonucleic acid (RNA). The liver from each animal was separated by differential centrifuging into a nuclear fraction and a cytoplasmic fraction. The latter was centrifuged to give a mitochondrial fraction, a fluffy layer, a microsomal fraction, and a supernatant fraction which was, in some experiments, further centrifuged to sediment an ultracentrifugal fraction.

2. Hypophysectomized rats showed a decreased recovery of labelled leucine in cytoplasmic protein, each of the fractions sharing in this decrease. The recovery tended to be even lower after growth-hormone treatment in the case of the cytoplasm as a whole and of the microsomal fraction.

3. Adrenalectomized rats showed a decreased recovery of leucine in the protein of the cytoplasm as a whole and of the microsomal fraction, but the decrease was smaller than that observed after hypophysectomy.

4. Hypophysectomized rats showed a decreased recovery of labelled orotic acid in the RNA of the mitochondrial fraction, the fluffy layer, and the microsomal fraction. These effects of hypophysectomy were not significantly influenced by growth hormone.

5. Adrenalectomized rats showed a decreased recovery of orotic acid in the RNA of the microsomal fraction, but an increased recovery in that of the cytoplasm as a whole, of the supernatant fraction, and of the ultracentrifugal fraction.

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The Metabolism of the Monochloronitrobenzenes in the Rabbit

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The results of a detailed study of the metabolism of tetra- and penta-chloronitrobenzenes in the rabbit (Bray, Hybs, James & Thorpe, 1953; Betts, James & Thorpe, 1955) were of sufficient interest to justify an investigation of the metabolic fate of the complete series of chloromononitrobenzenes. One feature of the metabolism of these polychloronitrobenzenes which particularly attracted our attention was that the mercapturic acids appeared to be formed by replacement of the nitro group by an acetylcysteyl group (acetylcysteyldenitration) and not by the usual process which is, in effect, an acetylcysteyldeprotonation and which occurs, for example, with naphthalene (Bourne & Young, 1934; Ing, Bourne & Young, 1934; Stekol, 1935), anthracene (Boyland & Levi, 1936) and the mono-

halogenobenzenes (Jaffe, 1879; Baumann & Preusse, 1879; Baumann & Schmitz, 1895; Young & Zbarsky, 1944). Another feature was the failure to detect any excretion of chloroacetanilides, although comparatively large amounts of polychloroanilines were excreted. A preliminary examination of di- and tri-chloronitrobenzenes (Bray, James & Thorpe, 1955*a*, *b*) has revealed a similar excretion of the corresponding unconjugated chloroanilines and, in most cases, the formation of mercapturic acids. These mercapturic acids were, however, formed, in effect, by acetylcysteyldechlorination, and not by replacement of the nitro group.

The present paper describes the effect of administration of *o*-, *m*- and *p*-chloronitrobenzene on the excretion by the rabbit of glucuronic acid, ethereal sulphate, mercapturic acid and diazotizable amines. Several phenolic metabolites have been identified by paper chromatography. The monochloronitrobenzenes are too toxic to permit the administration of large doses.

MATERIALS

Melting points of all compounds are uncorrected and agree with those given in the literature unless stated otherwise. The chloronitrobenzenes and chloroanilines were purchased. Chloroacetanilides (m.p. *o* 87°, *m* 72°, *p* 178°) were prepared by acetylation of the corresponding anilines with acetic anhydride. The methods of preparation and melting points of the chloronitrophenols and aminochlorophenols which are possible metabolites of the chloronitrobenzenes are given in Table 1. All aminochlorophenols were prepared by reduction of the corresponding nitro compounds with alkaline $Na_2S_2O_4$. Only the following require comment.

3-Amino-2-chlorophenol. Schlieper (1893) gave m.p. 85–87° for an impure specimen. Our material had m.p. 110–111°. (Found: N, 9·8. Calc. for C_6H_6ONCl: N, 9·8%.)

3-Chloro-5-nitrophenol. The method of Hodgson & Wignall (1926) was modified by using H_2S in ethanolic ammonia instead of sodium trisulphide for the reduction of 3:5-dinitroanisole. The experimental details given by Hodgson & Wignall for the demethylation of 3-chloro-5-nitroanisole are not clear. When the sulphuric acid solution is poured on to ice the desired product is left in solution and not in the precipitate, which is the unchanged anisole.

2-*Amino*-6-*chlorophenol*, m.p. 74°, is unstable. It gave a red–brown colour with $FeCl_3$. The diacetyl derivative, 2-*acetamido*-1-*acetoxy*-6-*chlorobenzene*, m.p. 128°, formed long colourless needles from water. (Found: C, 53·2; H, 4·4; N, 6·2. $C_{10}H_{10}O_3NCl$ requires C, 52·8; H, 4·4; N, 6·2%.)

3-*Amino*-5-*chlorophenol*, colourless needles, m.p. 129°, which rapidly turned black in air, gave a brown colour with $FeCl_3$. 3-*Acetamido*-5-*chlorophenol*, m.p. 161°, formed colourless prisms from aqueous ethanol. (Found: N, 7·8. $C_8H_8O_2NCl$ requires N, 7·6%.)

5-Amino-2-chlorophenol, m.p. 158°, colourless needles from aqueous ethanol, gave a brown colour with $FeCl_3$. (Found: C, 50·2; H, 4·0; N, 9·6; Cl, 24·3. Calc. for C_6H_6ONCl: C, 50·2; H, 4·2; N, 9·8; Cl, 24·7%.) (This compound is mentioned by Borowski (1951) but no properties or method of preparation are given.)

Mercapturic acids were prepared according to the method of Parke & Williams (1951). *p*-Nitroaniline was diazotized by adding 5 g. to a solution of $NaNO_2$ (2·5 g.) in conc. H_2SO_4 (20 ml.) at room temp. The mixture was diluted with an equal volume of water, with cooling, before addition to the cuprous mercaptide solution. *N*-*Acetyl*-*S*-(2-*nitrophenyl*)-L-*cysteine* had m.p. 156–158°, $[\alpha]_D^{18}+91\pm4°$ in ethanol (*c*, 0·55); $\lambda_{max.}$ 245, 375 mμ.; $\epsilon_{max.}$ 13 450, 2890 in 0·1 N-KOH. (Found: C, 46·3; H, 4·5; N, 9·9; S, 11·6%; equiv. 293. $C_{11}H_{12}O_5N_2S$ requires C, 46·5; H, 4·3; N, 9·9; S, 11·3%; equiv. 284.) *N*-*Acetyl*-*S*-(4-*nitrophenyl*)-L-*cysteine* had m.p. 156–158°, $[\alpha]_D^{26}+3\pm6°$ in ethanol (*c*, 0·33); $\lambda_{max.}$ 347 mμ.; $\epsilon_{max.}$ 10 230 in 0·1 N-KOH. (Found: C, 46·3; H, 4·1; N, 9·9; S, 11·2%; equiv. 287. $C_{11}H_{12}O_5N_2S$ requires C, 46·5; H, 4·3; N, 9·9; S, 11·3%; equiv. 284.)

METHODS

Animals, diet and dosage. Doe rabbits (2–3 kg. body wt.) were maintained on a constant diet of rabbit pellets and water (Bray, Ryman & Thorpe, 1947). Dose levels were 0·1 g./kg. for *o*-chloronitrobenzene and *o*-, *m*- and *p*-chloroanilines and 0·2 g./kg. for *m*- and *p*-chloronitrobenzenes. No toxic effects were observed at these levels, but larger doses caused severe anorexia and were sometimes fatal. For most purposes urines were collected each 24 hr. until metabolites were no longer excreted (usually 48 hr.). To minimize confusion with base-line values some urines, particularly for mercapturic acid determinations, were collected as separate consecutive samples from rabbits given water at intervals (see Bray, Thorpe & White, 1951).

Ether glucosiduronic acid was determined by a naphthoresorcinol method and ethereal sulphate by a turbidimetric method (see Bray & Thorpe, 1954). Mercapturic acid was determined by a modification (Betts *et al.* 1955) of the method of Stekol (1936); the concentration of NaOH used for the hydrolysis was 2 N. Recovery of added *N*-acetyl-*S*-(2-nitrophenyl)-L-cysteine from water was 81±3% and of *N*-acetyl-*S*-(4-nitrophenyl)-L-cysteine 91±3%: corresponding recoveries from urine were 44±3 and 63±3% respectively. *N*-Acetyl-*S*-(4-nitrophenyl)-L-cysteine was also determined in urine colorimetrically. Urine (5 ml.) diluted with 20 ml. of water was treated with 10% (w/v) $ZnSO_4,7H_2O$ (5 ml.) and 0·5 N-NaOH (5 ml.), mixed and filtered. A 10 ml. sample of the filtrate was heated with 1 N-NaOH (1 ml.) for 20 min. in a boiling-water bath, with a 'cold finger' to reduce evaporation. The absorption of the cooled yellow solution was measured in a Spekker photoelectric absorptiometer with an Ilford filter, spectrum violet 601 (max. transmission 430 mμ.). The calibration curve for pure *N*-acetyl-*S*-(4-nitrophenyl)-L-cysteine was linear, and a drum reading of 0·1 corresponded to 5 μg./ml. [This method is not applicable to *N*-acetyl-*S*-(2-nitrophenyl)-L-cysteine, which gives only a feeble yellow colour after heating with NaOH.] Urines from animals dosed with *p*-chloronitrobenzene contained small amounts (equivalent to less than 1% of the dose on any day) of chloronitrophenols, which give a yellow colour with NaOH in the cold. Correction for this was made by measuring the absorption immediately after addition of NaOH and subtracting this value from that obtained after heating. The average recovery of the mercapturic acid added to urine was 85±5%.

The method of Bratton & Marshall (1939) was used for determination of amino compounds. In the absence of

Table 1. *R_F values and detection of some possible metabolites of* o-, m- *and* p-*chloronitrobenzenes*

Solvent mixtures and time of runs on Whatman no. 4 paper: *A*, light petroleum (b.p. 90°) saturated with 98% formic acid (1 hr.); *B*, light petroleum (b.p. 90°)–benzene–98% formic acid (3:1:2, by vol.) (1 hr.); *C*, chloroform–acetic acid–water (2:1:1, by vol.) (2·2 hr.); *D*, benzene–acetic acid–water (2:2:1, by vol..) (1·5 hr.); *E*, *n*-butanol–3 N-$(NH_4)_2CO_3$–3 N-NH_3 soln. (4:3:3, by vol.; Corner & Young, 1954) (2 hr.); *F*, 96% (v/v) ethanol–water–NH_3 soln. (sp.gr. 0·88) (20:4:1, by vol.) (2 hr.). Detecting reagents: *a*, 2 N-HCl, 0·1% $NaNO_2$, 0·5% ammonium sulphamate and 0·1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Bratton & Marshall, 1939); *b*, diazotized *p*-nitroaniline followed by 20% (w/v) Na_2CO_3 (Bray *et al.* 1950); *c*, 20% (w/v) Na_2CO_3; *d*, ammoniacal $AgNO_3$; *e*, ammonia in solvents *E* and *F*. Colours of spots: B, blue; Bn, brown; G, green; Gy, grey; M, magenta; Mv, mauve; Oc, ochre; Ol, olive green; O, orange; P, purple; R, red; V, violet; d, deep; p, pale. Spots of *o*-nitrophenol are fugitive, since this compound readily evaporates, and should be examined before drying the paper.

References to preparation of compounds: 1, Erp (1930); 2, Hodgson & Moore (1925); 3, Hodgson & Kershaw (1928); 4, this paper; 5, Musajo & Minchilli (1940); 6, Barton, Linnell & Senior (1945); 7, Hodgson & Wignall (1926); 8, Mottier (1934); 9, Christiansen (1923). Where observed melting points differ from those given in the literature the latter are given in parentheses.

Ref.	Compound	M.p.	R_F values in solvent mixture						Colour with detecting reagents				
			A	*B*	*C*	*D*	*E*	*F*	*a*	*b*	*c*	*d*	*e*
	Possible metabolites of *o*-chloronitrobenzene												
1	2-Chloro-3-nitrophenol	120·5°	0·13	0·75	—	—	0·69	0·78	—	—	dY	—	pY
2	3-Chloro-2-nitrophenol	41 (37·5)	0·80	1·0	—	—	0·69	0·83	—	—	dY	—	dY
2	3-Chloro-4-nitrophenol	121–122	0·02	0·07	—	—	0·69	0·80	—	—	Y	—	Y
1	4-Chloro-3-nitrophenol	126·5	0·02	0·14	—	—	0·87	0·82	—	—	dY	—	pY
	o-Nitrophenol	—	—	1·0	—	—	0·57	0·90	—	—	dY	—	pY
3	2-Amino-3-chlorophenol	122	—	—	0·95	0·80	—	—	Y	VB	—	G	—
4	3-Amino-2-chlorophenol	110–111	—	—	0·91	0·70	—	—	M	M	—	B	—
5	3-Amino-4-chlorophenol	151	—	—	0·83	0·54	—	—	M	P	—	Bn	—
3	4-Amino-3-chlorophenol	158 (159·5)	—	—	0·67	0·23	—	—	B	Oc	P	B	—
	o-Chloroaniline	—	—	—	1·0	1·0	—	—	M	Y	—	—	—
	o-Aminophenol	—	—	—	0·17	0·05	—	—	—	—	—	Ol	—
4	*N*-Acetyl-*S*-(2-nitrophenyl)-L-cysteine	—	—	0·0	—	—	0·39	0·77	—	—	—	—	Y
	Possible metabolites of *m*-chloronitrobenzene												
6	2-Chloro-4-nitrophenol	111	0·1	0·53	—	—	0·66	0·84	—	—	Y	—	Y
6	2-Chloro-6-nitrophenol	70	1·0	1·0	—	—	0·60	0·80	—	—	YO	—	dY
7	3-Chloro-5-nitrophenol	147	0·04	0·16	—	—	0·84	0·82	—	—	dY	—	pY
8	4-Chloro-2-nitrophenol	86–87	0·95	1·0	—	—	0·66	0·80	—	—	O	—	O
	m-Nitrophenol	—	—	0·09	—	—	0·81	0·80	—	—	dY	—	pY
8	2-Amino-4-chlorophenol	138	—	—	0·62	0·45	—	—	Mv	GyBn	—	Bn	—
4	2-Amino-6-chlorophenol	74	—	—	0·93	0·80	—	—	Y	GyBn	—	Bn	—
4	3-Amino-5-chlorophenol	129	—	—	0·51	0·26	—	—	M	O	—	Bn	—
9	4-Amino-2-chlorophenol	151 (153)	—	—	0·45	0·10	—	—	B	Bn	PBn	Gy	—
	m-Chloroaniline	—	—	—	1·0	1·0	—	—	M	Y	—	—	—
	m-Aminophenol	—	—	—	0·4	0·04	—	—	—	—	—	Bn	—
	Possible metabolites of *p*-chloronitrobenzene												
1	2-Chloro-5-nitrophenol	119	0·11	0·50	—	—	0·69	0·82	—	—	dY	—	pY
2	5-Chloro-2-nitrophenol	32 (41)	1·0	1·0	—	—	0·66	0·77	—	—	O	—	Y
	p-Nitrophenol	—	—	0·02	—	—	0·59	0·72	—	—	Y	—	Y
3	2-Amino-5-chlorophenol	152 (154)	—	—	0·51	0·13	—	—	GyB	P	—	Bn	—
4	5-Amino-2-chlorophenol	158	—	—	0·70	0·31	—	—	B	RP	—	Bn	—
	p-Chloroaniline	—	—	—	1·0	1·0	—	—	M	Y	—	—	—
	p-Aminophenol	—	—	—	0·2	0·0	—	—	—	—	—	Gy	—
4	*N*-Acetyl-*S*-(4-nitrophenyl)-L-cysteine	—	—	0·0	—	—	0·42	0·79	—	—	—	—	dY

certain identification of acetylated amines, the increase obtained after hydrolysis is referred to in Table 2 as conjugated chloroaniline. Nitro compounds were reduced (Bray, Thorpe & Wood, 1949) and determined as amino compounds.

Faeces passed during two consecutive 24 hr. periods after the administration of the compounds were ground, suspended in water and steam-distilled; nitro and amino compounds were determined in the distillates.

Paper chromatography. The procedure was as described by Bray, Thorpe & White (1950). The solvents and detecting reagents used and the R_F values of reference compounds are given in Table 1. For mercapturic acids, in addition to comparison of the colours and R_F values of the spots with those of synthetic specimens, the appropriate zones were cut out and eluted with water. The presence of sulphur in the dried eluates was confirmed by fusion with sodium.

Qualitative examination of urines. The general procedure for the urines collected from rabbits dosed with chloronitrobenzenes was to prepare ether extracts A, B, C and D.

Extract A. Urine as collected (pH 7–8) was continuously extracted with ether for 24 hr. The extract contained chloroanilines and unconjugated aminochlorophenols and traces of chloronitrophenols.

Extract B. The residual urine from the preparation of extract A was adjusted to pH 1 and re-extracted with ether. This extract contained unconjugated chloronitrophenols and mercapturic acids.

Extract C. The urine remaining after the preparation of extract B was hydrolysed by refluxing for 1 hr. with an equal volume of 10N-H_2SO_4 and then continuously extracted with ether. This extract contained chloronitrophenols present in the original urine as conjugates.

Extract D. The residual urine from the preparation of extract C was adjusted to pH 7 and extracted with ether. This extract contained aminochlorophenols originally present as conjugates.

Extract E. Urines from rabbits dosed with chloroanilines were hydrolysed by refluxing with an equal volume of 10N-H_2SO_4 for 1 hr., adjusted to pH 7 and continuously extracted with ether. The extract contained chloroanilines and aminochlorophenols originally present, free or conjugated. This procedure was also used for isolation of 2-amino-5-chlorophenol as a metabolite of *p*-chloronitrobenzene.

Isolation of metabolites

Chloroanilines. The urine was adjusted to pH 10 and steam-distilled. The distillates were continuously extracted with ether. The residue left after removal of solvent from the extract was treated with acetic anhydride. The melting points of the chloroacetanilides isolated were unchanged by admixture with authentic samples.

2-Amino-5-chlorophenol. An extract E was prepared from the urine of rabbits dosed with *p*-chloronitrobenzene and applied to a cellulose powder (Whatman standard grade) column (45 cm. long, 3 cm. diameter). Metabolites were eluted by the organic phase of a mixture of chloroform–acetic acid–water (3:1:1, by vol.) and the fractions collected were examined by paper chromatography. *p*-Chloroaniline moved with the solvent front and was separated. 2-Amino-5-chlorophenol was then eluted and purified by recrystallization from aqueous ethanol (50%, v/v). It had m.p. 150°, and, mixed with an authentic specimen (m.p. 152°), 151°. The yield was 20 mg. from an extract corresponding to a dose of 500 mg. of *p*-chloronitrobenzene.

RESULTS

The average daily excretions of normal metabolites by the rabbits used were of the same order as those found previously (e.g. Bray *et al.* 1953). The results obtained from the quantitative analysis of urines are summarized in Table 2. For each compound nearly the whole of the dose accounted for was excreted in urine as the chloroaniline or derivatives of phenolic metabolites. The values given for the total percentage of the dose accounted for do not include small amounts of phenolic metabolites (probably equivalent to less than 5% of the dose) which are excreted unconjugated (Table 3, extracts A and B). The values for mercapturic acid from *m*-chloronitrobenzene are of doubtful significance since the increases observed after administration of

Table 2. *Excretion of metabolites of* o-, m- *and* p-*chloronitrobenzenes by the rabbit*

Results are expressed as percentages of the dose, given as means with ranges in parentheses; superior figures indicate the number of experiments. Consecutive urine samples were analysed until excretion of metabolites ceased. The unabsorbed material found in faeces was completely reduced to the chloroaniline except in the case of the *para*-isomer, when it consisted of approximately 1 part of *p*-chloronitrobenzene and 2 parts of *p*-chloroaniline. The values for chloroanilines were obtained from steam-distillates of the pooled urines of six rabbits.

Chloronitrobenzene administered	*Ortho*	*Meta*	*Para*
Unabsorbed	0·3 (0·1, 0·5)[2]	0·6 (0·5, 0·7)[2]	2·8 (2·4, 3·2)[2]
Ether glucuronide	42 (26–56)[3]	33 (17–58)[9]	19 (9–27)[4]
Ethereal sulphate	24 (18–31)[3]	18 (4–30)[6]	21 (15–37)[4]
Mercapturic acid	7 (0–18)[3]	1 (0–1)[5]	7 (2–11)[6]*
			3 (0–19)[9]†
Chloroaniline, free	9	11	9
Chloroaniline, conjugated	0	0	4
Total accounted for	82	64	63

* Colorimetric method. This method was more sensitive than the Stekol method and indicated that small amounts (1% of dose) were excreted on the fourth day after dosage.
† Values by modified Stekol method.

the dose were, in general, within the range of values obtained from normal urines. Furthermore, the presence of a mercapturic acid was not detected on paper chromatograms. The values for *o*- and *p*-chloronitrobenzene by the Stekol method have been corrected according to the recoveries obtained when the synthetic mercapturic acids were added to urine (see Methods). That these do largely represent mercapturic acids arising from the doses given is supported by the detection of the respective mercapturic acids on paper chromatograms (see below). In the case of the *para*-isomer, the colorimetric method provides additional support. The values by this method are probably more reliable than those from the Stekol method since tne colour obtained with normal urine is negligible. Unchanged chloronitrobenzenes were apparently absent from the urines, since the amounts of chloroanilines found were not increased after reduction with zinc and hydrochloric acid.

The metabolites identified by paper chromatography are listed in Table 3. The presence of 3-chloro-4-nitrophenol in B extracts of urines from rabbits dosed with *o*-chloronitrobenzene was confirmed by identification of 4-amino-3-chlorophenol in a similar extract which had been treated with alkaline sodium dithionite. There was no evidence for loss of chlorine from any of the chloronitrobenzenes since the corresponding aminophenols were not detected in extracts A and D or, after reduction, in extracts B and C. No metabolites giving a green (catechol) colour with ferric chloride were detected. No spots corresponding to acetamidophenols were detected on the paper chromatograms.

Attempted isolation of mercapturic acids. Purified ether extracts B were chromatographed on hydrocellulose columns eluted with solvent *E*. Although fractions containing mercapturic acids (identified by paper chromatography) were obtained from urines of rabbits dosed with *o*- and *p*-chloronitrobenzene, no pure mercapturic acids were isolated.

DISCUSSION

The main metabolic processes undergone by the chloronitrobenzenes in the rabbit are reduction and hydroxylation. The phenols formed are excreted mainly as conjugates with sulphuric and glucuronic acids, and there is little or no acetylation of the chloroanilines formed. The hydroxylation products formed from chloronitrobenzenes and chloroanilines (Fig. 1) suggest that hydroxylation could precede or follow reduction of the nitro compound in most cases. Chloronitrophenols and aminochlorophenols were identified as metabolites of all the chloronitrobenzenes. Not all these aminochlorophenols, however, were found as metabolites of the corresponding chloroanilines, although all the aminophenols formed as metabolites of the chloroanilines were found to be metabolites of the chloronitrobenzenes. It seems likely that the

Table 3. *Metabolites of chloronitrobenzenes and chloroanilines detected by paper chromatography of urine extracts*

Compounds in parentheses were present in traces. For preparation of extracts see Methods section. Compounds in extracts C and D are probably excreted as conjugates.

Identified in extract	*Ortho*	*Meta*	*Para*
	After dosage with chloronitrobenzenes		
A	*o*-Chloroaniline	*m*-Chloroaniline	*p*-Chloroaniline
	3-Amino-4-chlorophenol	2-Amino-4-chlorophenol	2-Amino-5-chlorophenol
	4-Amino-3-chlorophenol	4-Amino-2-chlorophenol	—
	(2-Chloro-3-nitrophenol)	—	—
	(4-Chloro-3-nitrophenol)	—	—
	(3-Amino-2-chlorophenol)	—	—
B	3-Chloro-4-nitrophenol	2-Chloro-4-nitrophenol	(2-Chloro-5-nitrophenol)
	(3-Chloro-2-nitrophenol)	—	—
	N-Acetyl-*S*-(2-nitrophenyl)-L-cysteine	—	*N*-Acetyl-*S*-(4-nitrophenyl)-L-cysteine
C	3-Chloro-4-nitrophenol	2-Chloro-4-nitrophenol	(2-Chloro-5-nitrophenol)
	(2-Chloro-3-nitrophenol)	—	—
D	3-Amino-4-chlorophenol	2-Amino-4-chlorophenol	2-Amino-5-chlorophenol
	4-Amino-3-chlorophenol	4-Amino-2-chlorophenol	—
	(2-Amino-3-chlorophenol)	—	—
	(3-Amino-2-chlorophenol)	—	—
	After dosage with chloroanilines		
E	*o*-Chloroaniline	*m*-Chloroaniline	*p*-Chloroaniline
	4-Amino-3-chlorophenol	2-Amino-4-chlorophenol	2-Amino-5-chlorophenol
	(2-Amino-3-chlorophenol)	4-Amino-2-chlorophenol	—

2-amino-4-chlorophenol from *m*-chloronitrobenzene and the 2-amino-5-chlorophenol from *p*-chloronitrobenzene are formed by hydroxylation of the chloroanilines, since the corresponding chloronitrophenols were not detected. On the other hand, the 3-amino-2-chloro- and 3-amino-4-chloro-phenol formed from *o*-chloronitrobenzene appear to have been formed by reduction of the corresponding chloronitrophenols, since the aminochlorophenols were not detected after administration of *o*-chloroaniline. Reduction of monochloronitrobenzenes in the rabbit seems to take place less readily than with some polychloronitrobenzenes, after administration of which no nitro compounds could be detected in urine (Bray *et al.* 1953; Betts *et al.* 1955). How much reduction occurs in the gut before absorption is not known.

With regard to the position of hydroxylation, the metabolites of the chloroanilines are all hydroxylated, as would be expected, either *ortho* or *para* to the amino group, and it is not surprising that 3-amino-2-chloro- and 3-amino-4-chloro-phenol, products of *meta* hydroxylation, were not found as metabolites of *o*-chloroaniline. Four nitrophenols were detected as metabolites of *o*-chloronitrobenzene. Two of these appear to have been formed contrary to the usual *ortho*- or *para*-direction of a chloro group and *meta*-direction of a nitro group. Robinson, Smith & Williams (1951), however, showed that in the rabbit nitrobenzene can be oxidized to *o*-, *m*- and *p*-nitrophenols. It may be relevant that the chloro group is anomalous in that, while being *ortho–para*-directing, it is deactivating (see Hammett, 1937). It might therefore be expected that the directing influence of a chloro and a nitro group together in the same nucleus might be complex.

Mercapturic acid formation by acetylcysteyldechlorination appears to provide a minor metabolic pathway for *o*- and *p*-monochloronitrobenzenes. This pathway does not seem to be available for *m*-chloronitrobenzene. Mercapturic acid formation in the chloromononitrobenzene series thus differs qualitatively as well as quantitatively from that in the chlorobenzene series. In the former, mercapturic acids are formed, in effect, either by acetylcysteyldenitration (Bray *et al.* 1953; Betts *et al.* 1955) or by acetylcysteyldechlorination (Bray *et al.* 1955*a*, *b*; present paper), whereas mercapturic acids from the chlorobenzene series are, in effect, products of acetylcysteyldeprotonation (Jaffe, 1879; Azouz, Parke & Williams, 1955; Parke & Williams, 1955; Jondorf, Parke & Williams, 1955).

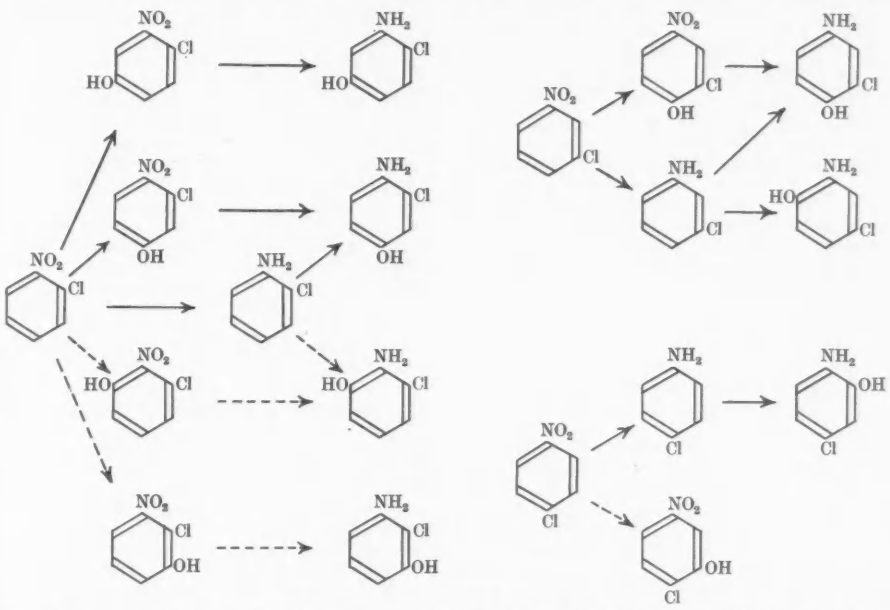

Fig. 1. Phenolic metabolites excreted (free or conjugated) in urine by the rabbit after dosage with *o*-, *m*- and *p*-chloronitrobenzene and *o*-, *m*- and *p*-chloroaniline. Broken arrows point to metabolites excreted only in very small amounts. (Although only a small amount of 4-chloro-3-nitrophenol was excreted, it is likely that a much greater amount was formed and reduced to 3-amino-4-chlorophenol before it was excreted.)

The extent of mercapturic acid formation in the chlorobenzene series appears to be greatest with monochlorobenzene, and only little, if any, is formed by the polychlorobenzenes (Jondorf *et al.* 1955). In contrast, very little mercapturic acid is formed by the monochloronitrobenzenes but considerable amounts are formed by many polychloronitrobenzenes.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-chloronitrobenzene has been studied in the rabbit within the limits imposed by the toxicity of these compounds.

2. The main products excreted in urine are phenols conjugated with glucuronic and sulphuric acids. Several amino- and nitro-chlorophenols have been identified by paper chromatography. 2-Amino-5-chlorophenol has been isolated from urine of rabbits dosed with *p*-chloronitrobenzene.

3. From all three isomers about 10% of the dose was excreted as free chloroaniline.

4. Small amounts of nitrophenylmercapturic acid are formed from *o*- and *p*-chloronitrobenzene.

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Some Chemical and Physiological Properties of 3-Indolylpyruvic Acid

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Stowe & Thimann (1953, 1954) claim to have demonstrated the presence of 3-indolylpyruvic acid (IPyA) in maize endosperm and consider (1953) that it may be identical with the accelerator-α of Bennet-Clark & Kefford (1953) and with other growth promoters described by Lexander (1953) and Terpstra (1953). Our work on the preparation, chemical and biological properties and chromatographic behaviour on paper of IPyA indicates that the presence of IPyA cannot be demonstrated by the methods used by Stowe & Thimann, and that it differs from accelerator-α in its effect on root growth.

EXPERIMENTAL

Chemical

Methods for the preparation of IPyA described by Ellinger & Matsuoka (1920) and by Gränacher, Gerö & Schelling (1924) were found to be unsatisfactory.

All melting points are uncorrected and were determined on a Kofler block.

Preparation of IPyA. Indole-3-aldehyde was condensed with hydantoin to give indolylidene hydantoin (Boyd & Robson, 1935). A solution of indolylidene hydantoin (15·3 g.) in air-free 0·5N-NaOH (1 l.) was kept at 80–90° under N_2 for 20 hr. It was then cooled to 0°, treated with ice-cold 3N-H_2SO_4 (200 ml.) and extracted successively with 300 and 100 ml. of ether. The combined ether extracts were washed with water, dried over anhydrous Na_2SO_4, and the ether was boiled off under reduced pressure. The residue was treated with warm acetone (30 ml.), the solution filtered, and the filtrate treated with acetic acid (40 ml.). After leaving at 0° for 1 hr. the IPyA was filtered off, well washed with acetic acid, and dried at 100°/0·1 mm. Mg over KOH pellets. The pale-brown powder thus obtained is essentially pure IPyA, dec. 190–208°: the yield was 6·4 g. (46%). Further crystallization of this product from acetone–acetic acid (1:1), followed by a crystallization from acetone, gave pale-yellow leaflets (3·65 g.); light absorption: maxima at 2320 and 3280–3290 Å (ϵ 27300 and 20100 respectively) in ethanol, and at 2320 and 3280 Å (ϵ 27100 and 22600) in a mixture of N-HCl and ethanol (1:9). (Found: C, 64·8; H, 4·7; N, 6·8. Calc. for $C_{11}H_9O_3N$: C, 65·0; H, 4·5; N, 6·9%.) A colourless product can be obtained by two further crystallizations from acetone. Melting point cannot be used as a criterion of purity, for the compound appears to decompose with melting over a range which depends on crystal size and rate of heating; the purest specimen (finely powdered) decomposed with melting over the range 193–209° (at approx. 5°/min. rise in temp.).

Characterization of IPyA. Since the substance described by Ellinger & Matsuoka (1920) and Gränacher *et al.* (1924) as IPyA was inadequately characterized, it was necessary to show that the substance obtained by us had in fact this constitution.

Oxidative decarboxylation of IPyA. A solution of IPyA (0·03 g.) in 0·5N-NaOH (2 ml.) was treated with 15% (w/v) H_2O_2 (0·5 ml.). After 30 min. the solution was acidified with N-H_2SO_4, and extracted exhaustively with ether. The extract was dried over anhydrous $MgSO_4$, evaporated to dryness, and recrystallized from $CHCl_3$ as colourless plates, m.p. 165–167°, unchanged by admixture with an authentic sample of 3-indolylacetic acid.

Preparation of the methyl ester of IPyA. A solution of diazomethane in ether was added to IPyA (0·15 g.) until the yellow colour of diazomethane persisted. The solution was immediately evaporated under reduced pressure, and the crystalline residue crystallized from a mixture of equal quantities of ether and light petroleum, b.p. 40–60°, to give the ester (0·1 g.) as colourless needles. After recrystallization from $CHCl_3$ the *methyl 3-indolylpyruvate* had m.p. 156–158°, light-absorption max. 2300 and 3280 Å (ϵ 20500 and 21300 respectively) in 95% ethanol. (Found: C, 66·6; H, 5·1; N, 6·3. $C_{12}H_{11}O_3N$ requires C, 66·35; H, 5·1; N, 6·4%.) This compound reduced ammoniacal $AgNO_3$ immediately in the cold, and gave a purple colour with ethanolic $FeCl_3$.

Reduction of methyl 3-indolylpyruvate. Methyl 3-indolylpyruvate (0·1 g.) was dissolved in ethanol (10 ml.) and sodium borohydride (0·02 g.) added in portions with shaking. After allowing the mixture to stand for 1 hr., the excess sodium borohydride was decomposed with a few drops of dilute acetic acid. Ethanol was removed *in vacuo*, water (6 ml.) added, and the mixture extracted with ether. After drying over anhydrous $MgSO_4$ the ethereal extract was evaporated to give methyl 3-indolyllactate as a low-melting glass characterized by hydrolysis to 3-indolyllactic acid and by further reduction to 3-(3′-indolyl)propane-1:2-diol.

Hydrolysis of methyl 3-indolyllactate. Methyl 3-indolyllactate (0·1 g.) was refluxed for 30 min. with 3% NaOH solution (8 ml.). After cooling, acidification followed by extraction with ether yielded 3-indolyllactic acid, which crystallized from ethyl acetate as prisms, m.p. 146–147° (Bauguess & Berg, 1934, give 144–145°). (Found: C, 64·3; H, 5·4; N, 6·6. Calc. for $C_{11}H_{11}O_3N$: C, 64·4; H, 5·4; N, 6·8%.) The compound can be obtained directly but less conveniently by sodium amalgam reduction of IPyA.

Preparation of 3-(3′-indolyl)propane-1:2-diol. Methyl 3-indolyllactate (0·2 g.) was refluxed in ether with lithium aluminium hydride (0·07 g.) for 1½ hr. The reaction mixture was cooled and the excess of lithium aluminium hydride decomposed by addition of water. A portion of 10% (w/v) NaOH solution (10 ml.) was added, the ether layer separated, and the aqueous layer extracted three times with ether. The combined ether extracts were dried (anhydrous $MgSO_4$) and evaporated to dryness. The residue crystallized from benzene as colourless plates, m.p. 99°, undepressed on admixture with an authentic specimen prepared by osmium tetroxide hydroxylation of 3-allylindole, as described by Brown, Henbest & Jones (1952). Yield 0·08 g. (47%). (Found: C, 68·7; H, 6·6; N, 7·4. Calc. for $C_{11}H_{13}O_2N$: C, 69·0; H, 6·8; N, 7·4%.)

Paper chromatography

The solvent systems used were (*A*) *iso*propanol–NH_3 soln. (sp.gr. 0·880)–water (8:1:1, by vol.); (*B*) acetic acid–water (1:3, v/v). Whatman's no. 1 paper was used throughout. All runs were carried out at room temperature (approx. 15°), except where otherwise stated.

Reagents for spraying. (1) Ammoniacal $AgNO_3$ was made by adding 50 ml. of NH_3 soln. (sp.gr. 0·880) to 100 ml. of N-$AgNO_3$. (2) Ehrlich's reagent. A solution of 2 g. of *p*-dimethylaminobenzaldehyde in a mixture of 20 ml. of HCl (sp.gr. 1·18) and 80 ml. of ethanol. (3) Nitrous–nitric acid. 1 g. of KNO_2 dissolved in 20 ml. of HNO_3 (sp.gr. 1·42) was diluted to 200 ml. (4) Dilute Salkowski reagent. A mixture of 2 ml. of $FeCl_3,6H_2O$ (0·05M) and 100 ml. of 5% (w/v) $HClO_4$. (5) Concentrated Salkowski reagent. A mixture of 1 ml. of $FeCl_3,6H_2O$ (0·5M) and 50 ml. of 35% (w/v) $HClO_4$. This reagent was diluted with an equal volume of ethanol before spraying.

One-dimensional chromatography of IPyA. IPyA (2 mg.) was divided between 12 spots and run upwards in solvent *A* until the solvent front had moved 23 cm. The paper was cut into strips and each strip eluted with water (12 ml.) for the bio-assay (Fig. 1, Table 1).

Note. For the identification of spot 6 (Table 1) observed in the one-dimensional chromatogram, 3-indolylglycollic acid was prepared in alkaline solution without isolation. Ethyl 3-indolylglyoxylate (10 mg.) was dissolved in ethanol (1 ml.) and sodium borohydride (10 mg.) added. The solution was boiled for 1 min., cooled, and then made up to 2·5 ml. with N-NaOH.

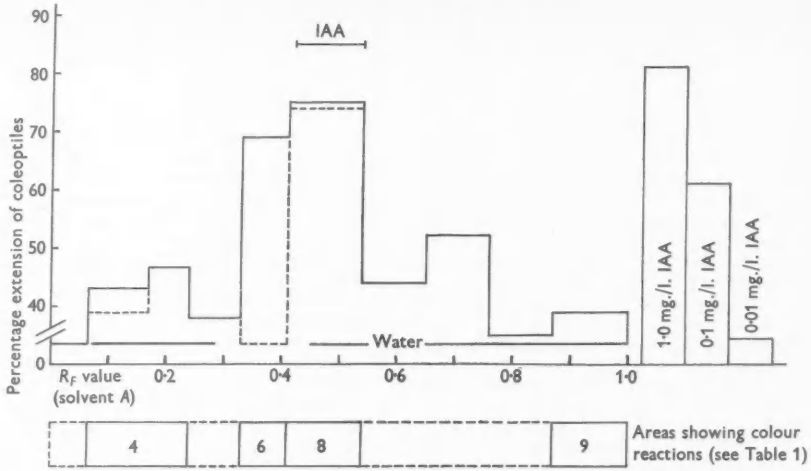

Fig. 1. Histogram of solvent *A* chromatogram of IPyA assayed on oats. Tenfold dilutions are indicated by broken lines.

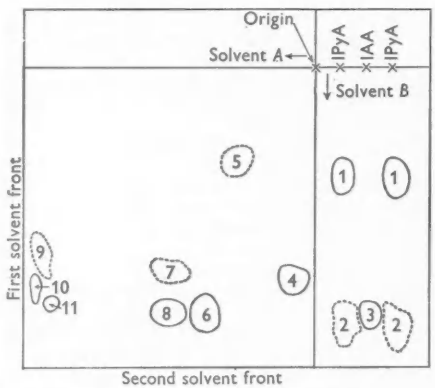

Fig. 2. Paper chromatogram obtained by developing IPyA in solvent *A* (after Stowe & Thimann, 1953), and then developing the decomposition products with fresh IPyA and 3-indolylacetic acid (IAA) as markers in solvent *B*. For key see Table 1. Broken lines indicate spots detectable only by fluorescence in ultraviolet light.

Two-dimensional chromatography of IPyA. IPyA (50–100 μg.) was chromatographed in solvent *A* until the solvent front had risen 20·3 cm. The paper was dried in a current of air for 30 min. at room temperature to remove NH_3. With solvent *B* at 27°, the decomposition products were now chromatographed in a direction at right angles to the original direction until the solvent front had risen 20·5 cm. 3-Indolylacetic acid (10–20 μg.) and a fresh sample of IPyA (10–20 μg.) were used as markers (Fig. 2, Table 1.)

Bio-assay techniques

Solutions to be tested for physiological activity were assayed by the *Avena* straight-growth method (Bentley, 1950; Bentley & Housley, 1954), except where otherwise stated. Briefly, the method consists of measuring the growth of isolated segments of oat coleoptiles in the solution to be tested. The assay is carried out at a constant temperature of 27° for approximately 18 hr., with 10 segments in 10 ml. of solution. At the stage of development at which the coleoptiles are used, growth occurs only by cell elongation.

The effect of IPyA on root growth was examined by a modified form of the technique of Moewus (1949). Cress seedlings, initial root length 5 mm., were grown on 1·5% agar into which the test solution had been incorporated. Growth was measured after approximately 18 hr. at 27°.

RESULTS AND DISCUSSION

Chromatography

Stowe & Thimann (1953, 1954) found that a growth-promoting constituent of maize (var. Country Gentleman) had R_F 0·12 in solvent *A* and gave a crimson colour with the concentrated Salkowski reagent. These authors chromatographed synthetic IPyA in solvent *A*, and on spraying with the concentrated Salkowski reagent found a crimson spot with R_F 0·12 compared with a similar spot of R_F 0·25 given by 3-indolylacetic acid under these conditions. On the basis of these experiments they identified the growth-promoting substance they had found in maize as IPyA.

Although Stowe & Thimann (1953, 1954) noticed some formation of 3-indolylacetic acid from IPyA

on chromatograms, and also found that on keeping their corn extracts for some time other breakdown products could be observed, they considered that IPyA could be chromatographed in solvent *A* without complete destruction. Our results on the chromatography of IPyA in solvent *A* are not in agreement with those of Stowe & Thimann, and show that IPyA is rapidly and completely broken down by this procedure.

Fig. 1 and Table 1 show the results of colour-spray tests and bio-assays on a one-dimensional chromatogram of IPyA in solvent *A*. Several physiologically active zones are formed, but no part of the chromatogram gives the immediate reduction of ammoniacal $AgNO_3$ in the cold, which is a characteristic reaction of IPyA. There is a region of rather low biological activity between R_F 0·065 and 0·24. Since this lies approximately half-way between the starting line and the 3-indolylacetic acid region (R_F 0·475 in this experiment), its position relative to 3-indolylacetic acid corresponds to that found for IPyA by Stowe & Thimann (1953).

With the spray reagents, part of the region between R_F 0·065 and 0·24 shows the colour reactions indicated in Table 1 (spot 4).

Spot 4 gives a yellow colour with the dilute Salkowski reagent, and a brownish yellow colour with the more concentrated Salkowski reagent used by Stowe & Thimann (1953). This indicates that spot 4 is not IPyA, which gives a purplish red colour with the concentrated Salkowski reagent.

When the decomposition products of IPyA from chromatography in solvent *A* are chromatographed in a second direction in solvent *B*, spot 4 has R_F 0·71, and no reducing zone is found with the R_F value of freshly applied IPyA (0·37). Solvent *B* causes very little breakdown of freshly applied IPyA. In this solvent, apart from the main IPyA spot (R_F 0·37, spot 1, Table 1), only one other spot can be distinguished (R_F 0·87, spot 2, Table 1). Spot 2 gives no reactions with any of the spray reagents and can only be detected because it shows a yellow fluorescence in ultraviolet light. The spray reagents give only one well-defined spot (spot 1) on solvent *B* chromatograms of IPyA, but sometimes a faint trailing pink colour can be seen between the IPyA region and the solvent front. This may be due to the formation of traces of 3-indolylacetic acid.

Since IPyA is stable in solvent *B*, any IPyA remaining after chromatography in solvent *A* would be expected to show up after further chromatography in solvent *B* as a reducing spot (R_F 0·37). Spot 5, which can be distinguished on the two-dimensional chromatogram, has R_F 0·31 in solvent *B* but cannot be IPyA since it gives no reaction with the spray reagents. It can only be detected because of its light-blue fluorescence in ultraviolet light.

Table 1. *R_F values and colour reactions observed in the chromatography of 3-indolylpyruvic acid (IPyA) (Figs. 1, 2)*

No. of spot	Ultraviolet fluorescence	Reaction with ammoniacal $AgNO_3$	Reaction with dilute Salkowski reagent	Reaction with nitrous–nitric acid mixture	Reaction with Ehrlich's reagent	R_F values (mean) Solvent *A*	Solvent *B*
1. IPyA	Bluish purple	Immediate reduction	Yellow with mauve border	Orange	Yellow changing to green	—	0·37
2.	Yellow	—	—	—	—	—	0·87
3. IAA*	Purple	Reduction overnight	Pink	Red	Mauve	0·50	0·83
4.	Orange–pink	Reduction during 10–20 min. after spraying	Very faint greyish yellow	Yellow	Faint green	0·07	0·71
5.	Light blue	—	—	—	—	0·27	0·31
6.†	Reddish mauve	Reduction after a variable period (15 min. to overnight)	Pink	Red	Pink	0·37	0·82
7.	Purple	—	—	—	—	—	0·68
8.	Purple	Reduction overnight	Pink	Red	Mauve	0·48	0·82
9.	Dark purple	—	Faint pink	Faint pink	—	0·93	0·63
10.	—	Very slight on long standing	—	—	Yellow changing to green	0·95	0·74
11.	—	Very slight on long standing	—	—	Mauve	0·89	0·79

* IAA: 3-indolylacetic acid.
† The intensity of the colour reactions given by spot 6 was reduced after running in solvent *B*.

Bio-assay of a two-dimensional chromatogram showed that spot 5 had no biological activity.

The region of the solvent *A* chromatogram showing the highest physiological activity is that associated with spots 6 and 8 (R_F 0·33–0·54). The zone corresponding to spot 8 (R_F 0·41–0·54) gives a solution with an absorption maximum at 280 mμ. and an intensity in agreement with an approximately 10% conversion of IPyA into 3-indolylacetic acid; the bio-assay results obtained with this zone are consistent with a 3-indolylacetic acid concentration of this order. The reactions of spot 8 with the spray reagents are completely consistent with the identification of this spot as 3-indolylacetic acid.

Bio-assays on solutions diluted tenfold show that the physiological activity of the spot 6 zone is less than one-tenth of that of the immediately adjacent spot 8 region. In an attempt to separate further the zones of activity associated with spots 6 and 8, IPyA was run downwards for 40 hr. in solvent *A*, but the results were essentially the same as those of Fig. 1. There was no clear-cut zone of lower activity between spots 6 and 8, and the possibility that the physiological activity associated with spot 6 is due to tailing of spot 8 is not ruled out.

Since spot 6 gives a red colour with the Salkowski reagent, it is possible that this is the spot observed by Stowe & Thimann (1953) between the starting line and the 3-indolylacetic acid region. However, the ratio of the R_F value of spot 6 to that of spot 8 is always close to 0·8 instead of approximately 0·5 as observed by Stowe & Thimann. Moreover, on chromatography in solvent *B*, spot 6 has R_F 0·82, whereas IPyA has R_F 0·37.

Spot 6 was compared with a number of indole compounds of similar R_F value. It differed from 3-indolylcarboxylic acid, 3-indolylacetic acid and 3-indolylglyoxylic acid in its colour reactions, but gave colour reactions identical in every respect with those of 3-indolylglycollic acid.

As described in the experimental part, 3-indolylglycollic acid was prepared and used in alkaline solution. This procedure is used because 3-indolylglycollic acid rapidly becomes red on acidification of its alkaline solutions. This decomposition in acid solution is shown by spot 6, which gives less intense colour reactions after chromatography in acetic acid solution, and a red tailing streak down the paper. The two growth-promoting zones between the 3-indolylacetic acid region and the solvent front (Fig. 1) show some faint colour reactions with the spray reagents, but have none of the reactions characteristic of IPyA.

It is evident that chromatography in solvent *A* decomposes IPyA completely, and it follows that the substance (R_F 0·12 in solvent *A*) shown by Stowe & Thimann to occur in maize cannot be IPyA; nor can the substance with similar properties observed by these authors in their solvent *A* chromatograms of IPyA be IPyA itself. It is possible that an impurity was present in Stowe & Thimann's preparation, which cannot have been as pure as our material, since they say that 'it darkens rapidly on exposure to air', whereas our crystalline IPyA appears unchanged after keeping in air in a stoppered tube at room temperature for some months.

We are of the opinion that the presence of IPyA in maize has not been convincingly demonstrated, and do not consider that the presence of IPyA in any biological extract could be shown by the chromatographic procedure of Stowe & Thimann (1953).

Bio-assays

To examine the effect of IPyA on cell elongation, bio-assays of IPyA without chromatography were carried out (Table 2). The IPyA was dissolved in a drop of ethanol to facilitate solution; low concentrations of ethanol have no effect on the bio-assay. It is seen from these results that solutions of pure IPyA have low activity, and if this is expressed in terms of equivalent 3-indolylacetic acid concentrations, then at optimum activity it is less than 1% of the 3-indolylacetic acid.

Since it had already been shown that IPyA broke down on chromatography in ammoniacal solution, it seemed possible that some breakdown might also be occurring in the test solutions of the assay. To investigate this possibility a chromatogram of IPyA was run in water over a period of 2 hr., and bio-assayed. Results (Fig. 3) clearly indicate that there is breakdown of IPyA even in water over a short period of time. There appears to be continuous production of a growth-promoting substance, most concentrated near the solvent front in a region having the same spray reactions and

Table 2. *Activity of* 3*-indolylacetic acid* (*IAA*) *and IPyA in the* Avena *straight-growth test*

Figures represent final length (mm.) of sections initially 10 mm. long.

		Concn. (mg./l.)							
Expt.	Compound	0	0·01	0·1	1·0	10	25	50	100
a	IAA	14·0	14·2	15·9	16·9	17·8	—	—	14·2
	IPyA		13·3	13·2	13·8	14·9	—	—	—
b	IAA	13·1	14·5	16·6	17·4	15·6	—	—	—
	IPyA		13·3	13·3	14·3	15·3	—	—	—
c	IAA	12·4	13·3	15·8	16·2	16·8	—	—	13·4
	IPyA		12·8	12·6	13·2	13·6	14·5	14·3	13·7

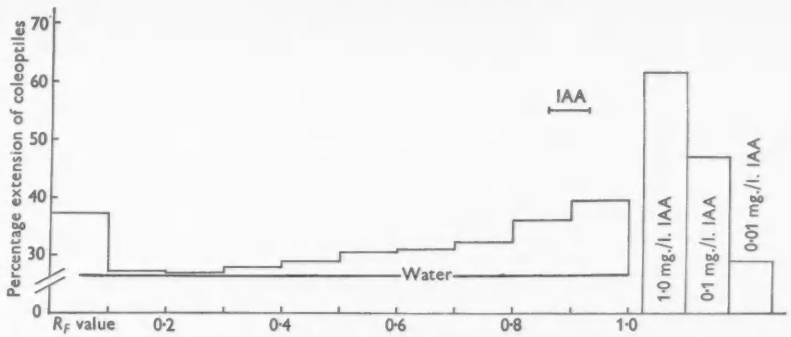

Fig. 3. Histogram of IPyA chromatographed in water for 2 hr.

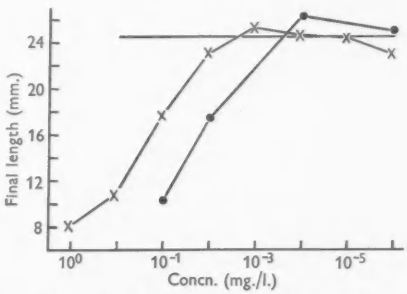

Fig. 4. Effect of 3-indolylacetic acid (IAA) ● and IPyA × on root growth. The horizontal line is at the final level given by a water control.

R_F value as 3-indolylacetic acid. On the starting line spray reactions characteristic of IPyA are shown: activity in this zone may be ascribed to continuous breakdown of unchanged IPyA during the bio-assay.

When IPyA was bio-assayed on roots, only inhibition of growth was obtained, the activity corresponding approximately to a 10% conversion into 3-indolylacetic acid (Fig. 4). This result shows clearly that the accelerator-α which promotes root growth (Bennet-Clark & Kefford, 1953) is not IPyA, as postulated by Stowe & Thimann (1953).

SUMMARY

1. 3-Indolylpyruvic acid (IPyA) has been synthesized by an improved method and fully characterized.

2. It has been shown to break down completely on chromatography under the conditions used to demonstrate its alleged presence in maize.

3. The physiological activity of synthetic IPyA in the *Avena* straight-growth test has been investigated and shown to be mainly, if not entirely, due to its breakdown products.

4. From an examination of its activity on root growth it has been demonstrated that it is not identical with accelerator-α.

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Studies in Detoxication

69. THE METABOLISM OF ALKYLBENZENES: *n*-PROPYLBENZENE AND *n*-BUTYLBENZENE WITH FURTHER OBSERVATIONS ON ETHYLBENZENE*

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An account of our studies on the metabolic fate of toluene, ethyl-, *iso*propyl- and *tert.*-butyl-benzenes has been given in earlier papers (Smith, Smithies & Williams, 1954*a*, *b*, *c*; Robinson, Smith & Williams, 1955; Robinson & Williams, 1955). The present paper deals with *n*-propyl- and *n*-butyl-benzenes. These two compounds were previously studied by Thierfelder & Klenk (1924), who showed that *n*-propylbenzene was oxidized in rabbits to yield benzoic acid (excreted as hippuric acid) and *n*-butylbenzene to yield phenylacetic acid (excreted as phenaceturic acid). It was suggested that about 60–65% of these compounds were oxidized to acids and it was concluded that these alkylbenzenes underwent ω-oxidation initially and then, by β-oxidation of the resulting ω-carboxylic acid, they were converted into benzoic or phenylacetic acid according to the number of carbon atoms in the alkyl side chain. However, Smith *et al.* (1954*a*) found that *n*-propyl- and *n*-butyl-benzenes gave rise to appreciable amounts of non-reducing conjugated glucuronic acids, and suggested that the alkyl side chains of these compounds were oxidized in other positions as well as the ω- to yield secondary alcohols which were excreted conjugated with glucuronic acid. Both propyl- and butyl-benzene could be expected to be oxidized at the α-position, since this is activated by its proximity to the benzene ring (cf. ethylbenzene, Smith *et al.* 1954*b*). Furthermore, it has been observed that alkyl side chains in barbiturates (Maynert, 1952) and the *n*-butyl chain in the antirheumatic drug, phenylbutazone (butazolidine) (Burns *et al.* 1955), are oxidized in the $(\omega-1)$-position. This type of oxidation could also occur with *n*-propyl- and *n*-butyl-benzene. It will, in fact, be shown here that both these compounds are oxidized in rabbits at the α- and $(\omega-1)$-positions of the alkyl side chain. Further observations on the metabolism of ethylbenzene have now shown that this compound undergoes ω-oxidation, although the major process is α-oxidation.

* Part 68: Parke (1956).

EXPERIMENTAL

Reference compounds. Secondary alcohols were identified by oxidizing them to ketones. The 2:4-dinitrophenylhydrazones of propiophenone, benzyl methyl ketone, butyrophenone, benzyl ethyl ketone and methyl 2-phenylethyl ketone were prepared (for references see Heilbron & Bunbury, 1953). The characteristics of the spectra of these compounds are given in Table 1. Methyl [(−)-ethylphenylcarbinyl tri-*O*-acetyl-β-D-glucosid]uronate, m.p. 127° and $[\alpha]_D^{20}-80°$ in $CHCl_3$, was a sample previously prepared (Smith *et al.* 1954*c*).

The azlactone of hippuric acid with *p*-dimethylaminobenzaldehyde has been described (Gaffney, Schreier, DiFerrante & Altman, 1954). Attempts to prepare the corresponding azlactone of phenaceturic acid were unsuccessful. The azlactone of cinnamoylglycine was prepared from *p*-dimethylaminobenzaldehyde and cinnamoylglycine in the presence of acetic anhydride in the usual manner. The 4-p-*dimethylaminobenzal*-2-(2-*phenylvinyl*)-5-*oxazolone* formed deep-red needles, m.p. 194°, from acetone. (Found: C, 75·1; H, 5·8; N, 8·7. $C_{20}H_{18}O_2N_2$ requires C, 75·4; H, 5·7; N, 8·8%; light absorption, $\lambda_{max.}$ 255, 320 and 490 mμ. with $\epsilon_{max.}$ 12000, 20000 and 61000 respectively in ethanol.)

Animals and diet. Chinchilla rabbits weighing 2·5–3·5 kg. were used throughout this work. They were kept on a constant diet consisting of Bruce & Parke's diet no. 18 (Associated London Flour Millers). Compounds were administered by stomach tube.

Determination of hippuric acid

Gaffney *et al.* (1954) described a micro-method which involves the formation of a coloured azlactone from hippuric acid and *p*-dimethylaminobenzaldehyde. Hippuric acid is separated from urine by paper chromatography and the azlactone is formed on the paper, then eluted for estimation. We have modified this procedure so that the estimation can be carried out on the urine without paper chromatography.

Reagents. Ethyl acetate (May and Baker, Ltd., 98% esters) and 7% (w/v) *p*-dimethylaminobenzaldehyde (A.R. British Drug Houses Ltd.) in acetic anhydride (May and Baker Ltd.; not less than 96% at assay) were used. The *p*-dimethylaminobenzaldehyde should not give any red colour on dissolving it in acetic anhydride and if necessary should be purified by crystallization from benzene. The concentration of *p*-dimethylaminobenzaldehyde necessary for optimum colour production had been determined in a separate experiment.

Determination in pure solution. Aqueous solutions of hippuric acid containing 50–250 μg./ml. were prepared and 1 ml. of each solution was shaken vigorously with 5 ml. of ethyl acetate in a 15 ml. centrifuge tube. The mixture was centrifuged for 2·5 min. and 1 ml. samples (in triplicate) of the clear extract were evaporated to dryness in 6 in $\times \frac{5}{8}$ in. test tubes in an oven at 110°. The dry residue (a trace of water here gives high results) in the tube was treated with 3 ml. of the *p*-dimethylaminobenzaldehyde solution and the tube was then heated in an oil bath at 120° for 0·5 hr. This temperature (which should be carefully controlled) and time had been found to give the best results in a separate experiment. The tube was cooled to room temp., 2 ml. of ethyl acetate was added to dilute the coloured product and the yellowish-brown colour of the solution was measured in a SP. 600 Unicam spectrophotometer at 460 mμ. against a blank test prepared similarly, except that water was substituted for the hippuric acid solution. The calibration curve was a straight line, and the error in the recovery of concentrations of hippuric acid from 150 to 250 μg./ml. ranged from $\pm 10\%$ at the lower level to $\pm 5\%$ at the higher concentrations.

Determination in rabbit urine. Urine collected during 24 hr. was brought to pH 2–3 with conc. HCl (about 0·5–2 ml.) and then diluted to 200 ml. The diluted urine (1 ml.) was then extracted with 5 ml. of ethyl acetate and treated as described above. The recovery of hippuric acid added to urine in quantities corresponding to 15–50 mg./day was within $\pm 10\%$. In one set of determinations on normal rabbit urine (113 samples on diet 18), the output of apparent hippuric acid was 20–81 mg./day (average, 43 mg.). Beer, Dickens & Pearson (1951) found that rabbits excreted 5–90 mg./day as benzoic acid on a cabbage diet.

Large excesses (100:1, w/w) of amino acids (glycine, alanine, serine, lysine, cystine, methionine, ornithine, histidine, tyrosine and tryptophan) added to the urine did not interfere. The effect of phenaceturic acid was irregular but it did not interfere if it were present in an amount not greater than half that of the hippuric acid. The interference from phenaceturic acid was less if the colour from hippuric acid were measured at 470 mμ. instead of 460 mμ. At both these wavelengths, the extinction of the hippuric acid colour is the same. When the estimation procedure is carried through with phenaceturic acid instead of hippuric acid, the resulting extract shows a relatively low maximum absorption at 435 mμ.

Paper chromatography of glycine conjugates

The separation of hippuric and phenaceturic acids (20 μg. or less in water) on paper was achieved with benzene–acetic acid–water (1:1:2, by vol.). Whatman no. 4 paper (descending chromatography) was used. The time of run was 5 hr. at room temp. The paper was then dried and sprayed with a 10% solution (w/v) of *p*-dimethylaminobenzaldehyde in acetic anhydride. When the paper was heated in an oven at 120° for 2 min. or before an electric fire, hippuric acid showed up as an orange-red spot (R_F 0·18) and the phenaceturic acid as a yellow spot (R_F 0·28). Cinnamoylglycine could also be separated from these compounds since under the same conditions it showed up as a deep-red spot (R_F 0·34). Phenylpropionylglycine also separated from hippuric and phenaceturic acids but it overlapped with cinnamoylglycine, and gave a bright-yellow spot (R_F 0·37). Mixtures of the first three conjugates were readily separated and the

Table 1. *Absorption spectra of the 2:4-dinitrophenylhydrazones of certain ketones*

			Spectra in ethanol			
			Authentic sample		Urinary sample	
2:4-Dinitrophenylhydrazone of	M.p.	Cryst. form	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.} \times 10^{-3}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.} \times 10^{-3}$
Ethyl phenyl ketone	191°	Red plates	375, ~ 260	26·4, 11·6	375, ~ 260	26·8, 13·0
Benzyl methyl ketone	153–154°	Deep-yellow needles	360, ~ 260	22·4, 11·0	360, ~ 260	22·7, 12·0
Phenyl propyl ketone	194–195°	Red plates	375, ~ 260	27·5, 13·0	375, ~ 260	27·5, 13·0
Benzyl ethyl ketone	145°	Deep-yellow needles	360, ~ 260	23·5, 12·0	—	—
Methyl 2-phenylethyl ketone	128°	Crimson prisms	360, ~ 260, 230	22·0, 10·7, 16·2	360, ~ 260, 230	22·0, 11·3, 16·5

components detected by this procedure in pure solution and in urine. The urine was first acidified to about pH 2, about 0·01 ml. was placed at the starting line, dried and then irrigated with the solvent. Normal rabbit urine gave a faint spot corresponding to hippuric acid, but none corresponding to phenaceturic acid. Rough estimates of the amounts of phenaceturic acid in a given urine were obtained by comparison of the urinary spot with those obtained from standard amounts of phenaceturic acid.

Isolation of metabolites

From ethylbenzene

Isolation of phenaceturic acid (see also Table 2). A total of 17·8 g. of ethylbenzene was fed to ten rabbits and the urine was collected for 2 days. The urine (1500 ml.) was acidified to pH 2 with 2N-H_2SO_4 and continuously extracted with ether for 16 hr. The extract was evaporated and the residue, which contained glucuronides, was dissolved in water (10 ml.) and kept overnight. The crystals (2·8 g.) which were deposited were washed with a few drops of water and dissolved in 25 ml. of hot water. From the solution at 30° crystals of hippuric acid (1·5 g.) were separated, m.p. and mixed m.p. 189–190°. (Found: N, 7·6. Calc. for $C_9H_9O_3N$: N, 7·8%.) Further cooling to 20° gave impure phenaceturic acid (0·8 g., m.p. 138–140°). After two recrystallizations from ethyl acetate, pure phenaceturic acid, m.p. and mixed m.p. 144°, was obtained. (Found: N, 7·3. Calc. for $C_{10}H_{11}O_3N$: N, 7·3%.) Further ether extraction of the urine for 2 days yielded a further 0·8 g. of mixed crystals, and the mother liquors from the first extraction 0·4 g. These fractions were mixed and the impure phenaceturic acid (m.p. 135–140°) was separated from them. This was then hydrolysed by boiling for 4 hr. with N-NaOH. On acidification phenylacetic acid separated, which on recrystallization from light petroleum (b.p. 60–80°) had m.p. and mixed m.p. 75–76°.

From n-*propylbenzene*

Isolation of ethylphenylcarbinyl glucosiduronic acid. A total of 16 ml. (13·8 g.) of *n*-propylbenzene was fed to eight rabbits and the urine was collected for 24 hr. It was acidified to about pH 2 with dilute H_2SO_4 and continuously extracted with ether for 60 hr. The extract was evaporated to a syrup which was triturated with a little water and kept overnight to allow hippuric acid to separate. The acid was filtered off and the filtrate evaporated to a gum. This was dissolved in 100 ml. of ether containing a little methanol and then poured into 500 ml. of light petroleum (b.p. 40–60°). The glucuronide gum (10 g.) which was precipitated was methylated with diazomethane and then acetylated with pyridine and acetic anhydride. The product on pouring into water gave 8 g. of crude triacetyl methyl esters. Fractional crystallization from absolute ethanol yielded 2·5 g. of pure methyl [(−)-ethylphenylcarbinyl tri-*O*-acetyl-β-D-glucosid]uronate, m.p. and mixed m.p. 125° and $[\alpha]_D^{20}$ − 82° in $CHCl_3$ (*c*, 1). (Found C, 58·3; H, 6·2. Calc. for $C_{22}H_{28}O_{10}$: C, 58·7; H, 6·2%.) Other small crystalline fractions were obtained with $[\alpha]_D$ − 60 to − 70° in $CHCl_3$, but the main residue was a gum with $[\alpha]_D$ − 27°, which was very soluble in ethanol and did not crystallize. This was suspected to contain methyl [(+)-ethylphenylcarbinyl tri-*O*-acetyl-β-D-glucosid]uronate, whose rotation would be expected to be about − 15°, and methyl [(+)- and (−)-benzylmethylcarbinyl tri-*O*-acetyl-β-D-glucosid]uronate, whose rotations are known to be − 24° and − 46° respectively (Smith *et al.* 1954*c*). We failed, however, to isolate the glucosiduronic acid of either form of benzylmethylcarbinol in a crystalline state, but proof of the presence of this carbinol was obtained by converting it into the ketone (see below).

Isolation of propiophenone and benzyl methyl ketone. The experiment described above was repeated to the stage of precipitating the glucuronide gum with light petroleum (the yield of hippuric acid, m.p. and mixed m.p. 186°, was 2·8 g. or 14·6% of the dose on the assumption that it was mainly from propylbenzene). The glucuronide gum (10 g.) was dissolved in a little water, treated with charcoal and filtered. The filtrate was diluted to 500 ml. with 0·1M acetate buffer of pH 4·6, and incubated for 48 hr. at 37° with 0·5 ml. of brown locust-crop liquor (Robinson, Smith & Williams, 1953) containing β-glucuronidase. The mixture was then made alkaline with solid K_2CO_3 and extracted with 3 × 100 ml. of ether. The extract was dried over anhydrous Na_2SO_4 and the ether was evaporated, leaving about 0·5 ml. of oily residue of carbinols. This was oxidized by keeping overnight in 10 ml. of pyridine containing 1 g. of chromium trioxide. The product was diluted with an excess of ice-cold 2N-HCl and the solution extracted with 3 × 50 ml. of ether. Evaporation of the ether left a small quantity of oil smelling of ketones. Benzyl methyl ketone reacts with sodium bisulphite, but propiophenone is inert. The oil was therefore treated with 2 ml. of saturated aqueous sodium bisulphite solution. After 5 hr. the small amount of crystals which had formed was filtered and converted into the 2:4-dinitrophenylhydrazone. After recrystallization from ethyl acetate, 10 mg. of the deep yellow 2:4-dinitrophenylhydrazone of benzyl methyl ketone was isolated, m.p. and mixed m.p. 153°. Its absorption spectrum ($\lambda_{max.}$ 360 mμ.) was identical with that of the authentic compound (see Table 1). (Found: N, 17·7. Calc. for $C_{15}H_{14}O_4N_4$: N, 17·8%.) The filtrate from the bisulphite compound contained the bulk of the ketonic material obtained in the oxidation. From it there was isolated the red plates of propiophenone 2:4-dinitrophenylhydrazone (0·5 g.), m.p. and mixed m.p. 191°, with an absorption spectrum ($\lambda_{max.}$ 375 mμ.) identical with the authentic compound. (Found: N, 17·9%.) The isolation of these compounds proved the presence of ethylphenylcarbinol and of benzylmethylcarbinol in the glucuronide gum, but gave no indication of their stereochemical form.

From n-*butylbenzene*

Isolation of phenaceturic acid, butyrophenone and methyl 2-phenylethyl ketone. Attempts at crystallizing the glucuronide fraction from the urine of rabbits dosed with butylbenzene failed. *n*-Butylbenzene (16 ml.) was fed as described above for *n*-propylbenzene, and the same procedure was followed. Instead of hippuric acid, phenaceturic acid was separated in a yield of 1·9 g. (10% of the dose) with m.p. and mixed m.p. 145° after recrystallization from water. The glucuronide gum (8 g.) was again obtained by the light-petroleum treatment and incubated with locust-crop liquor as above. About 1 ml. of carbinols was thus obtained, and this was oxidized with chromic oxide in pyridine. The methyl ketones were again separated as the bisulphite compounds and eventually 20 mg. of the crimson prisms (from ethyl acetate) of methyl 2-phenylethyl ketone 2:4-dinitrophenylhydrazone was obtained, m.p. and mixed m.p. 127° ($\lambda_{max.}$ 360 mμ.). (Found: N, 17·3. Calc. for $C_{16}H_{16}O_4N_4$:

N, 17·1%.) The ketonic material which did not react with bisulphite was converted into the 2:4-dinitrophenylhydrazone, and after recrystallization the red plates of butyrophenone 2:4-dinitrophenylhydrazone were obtained, m.p. and mixed m.p. 194° ($\lambda_{max.}$ 375 mμ). (Found: N, 17·1%.) The isolation of the two dinitrophenylhydrazones proved the presence of methyl-phenylethylcarbinol and phenyl-*n*-propylcarbinol in the glucuronide gum. Benzylethylcarbinol, the other possible metabolite of *n*-butylbenzene, was not detected.

Detection of p-*hydroxyphenylacetic acid.* Smith *et al.* (1954*a*) reported that rabbits receiving butylbenzene excreted ethereal sulphate corresponding to 5% of the dose. This did not occur with ethylbenzene and the figure of 2% ethereal sulphate reported for propylbenzene was probably not significant. It was therefore possible that butylbenzene underwent aromatic hydroxylation and a probable metabolite is, therefore, *p*-hydroxyphenylacetic acid. This acid has been reported to be a constituent of normal rabbit urine (Bray, Thorpe & White, 1950), in which it was identified chromatographically on paper by its R_F value and colour reactions. In the present work *n*-propanol–NH_3 soln. (sp.gr. 0·88) (7:3), amongst other solvent mixtures, was used with Whatman no. 1 or 4 paper (descending chromatography). In this solvent it had R_F 0·36 and separated well from other constituents reacting with the reagents. The spraying reagents used were diazotized *p*-nitraniline and diazotized sulphanilic acid (Bray *et al.* 1950) and a dilute aqueous solution of Brentamine Fast Blue B Salt (Imperial Chemical Industries Ltd.); the coloured spots obtained were bluish red, red, and yellowish red respectively.

The urine output for 24 hr. (100 ml.) of a rabbit which had received 2 ml. of butylbenzene orally was heated under reflux for 2 hr. with 30 ml. of conc. HCl. The cooled urine was extracted with 3 × 150 ml. of ether. The acids in the extract were transferred to 10% NaOH (2 × 15 ml.). The alkaline solution was acidified and extracted with ether. A concentrate of the last ether extract was used for paper chromatography. The same extraction method was used on normal rabbit urine. Normal rabbit urine gave weak but definite spots corresponding to *p*-hydroxyphenylacetic acid. After butylbenzene the spots appeared to be about ten times stronger than those found with normal urine. This strongly suggests that *p*-hydroxyphenylacetic acid is a minor metabolite of butylbenzene.

DISCUSSION

Metabolism of ethylbenzene. Ethylbenzene is an important intermediate in the production of the styrene used in the plastics industry. In the U.S.A. in 1954, the output of ethylbenzene was 800 million lb. weight, which is more than any other intermediate listed in the U.S. Tariff Commission's statistics (Anon. 1955). According to Browning (1953) no systematic injury from ethylbenzene in man has been reported, but its acute toxicity is slightly more than that of benzene. The metabolites of ethylbenzene are in general relatively non-toxic, and there is no evidence that it is metabolized to phenolic substances which could produce chronic toxic effects as in the case of benzene. Its main metabolites in rabbits are hippuric acid and methylphenylcarbinyl glucosiduronic acid, which are produced in roughly equal amounts and account for at least 60–70% of the dose. Its minor metabolites are mandelic acid (2%) and, as shown in this paper, phenaceturic acid (10–20%). The results of our investigations suggest the scheme shown in Fig. 1 for the metabolism of ethylbenzene; the major routes are indicated by thick arrows. The figures in Table 2 suggest that methylphenylcarbinol is the precursor of hippuric acid since approximately the same amount of this acid is excreted whether ethylbenzene or the carbinol, a known metabolite of ethylbenzene, is fed. Acetophenone is also a precursor of hippuric acid, but our earlier work (Smith *et al.* 1954*b*) has shown that this ketone is reduced to methylphenylcarbinol *in vivo*, and need not be included in the scheme. It was not detected in the breath or urine of rabbits receiving ethylbenzene. Mandelic acid is apparently a minor end product of ethylbenzene metabolism and is not converted into hippuric acid. It is also a known minor metabolite of methylphenylcarbinol (Thierfelder & Klenk, 1924) and is presumably produced from ethylbenzene via the carbinol. The main process of ethylbenzene metabolism is thus initially oxidation of the activated α-carbon atom. The actual mechanism of this initial oxidation is as yet unknown. However, oxidation of the ω-methyl group of ethylbenzene does take place to a small extent, since phenaceturic acid can be isolated from the urine. 2-Phenylethanol (see Table 2) and phenylacetaldehyde are presumably intermediates in its formation.

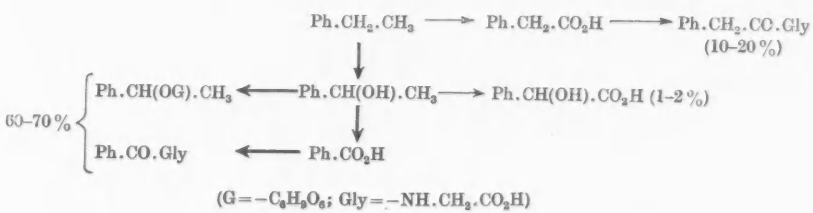

Fig. 1

Metabolism of n-*propylbenzene.* The metabolites of *n*-propylbenzene are hippuric acid, and the glucuronides of ethylphenylcarbinol and benzyl methylcarbinol. The results shown in Table 2 suggest the scheme shown in Fig. 2 for its metabolism.

What we believe to be major routes are indicated by thick arrows, minor routes by thin arrows, and hypothetical routes by broken arrows. Glucuronide and hippuric acid excretion account for about 60–70 % of the *n*-propylbenzene fed. Ethylphenylcarbinyl glucosiduronic acid can be readily isolated from the urine, but ethylphenylcarbinol itself is not readily converted into hippuric acid (see Table 2). Conjugated benzylmethylcarbinol was also detected as a metabolite of propylbenzene, but appeared to be present in the urine in lesser amounts than ethylphenylcarbinol. However, benzylmethylcarbinol is more readily converted into hippuric acid than the α-carbinol. It is to be noted that ethyl phenyl ketone and benzyl methyl ketone are equivalent to the carbinols as far as hippuric acid production is concerned. We conclude therefore that the degradation of propylbenzene to hippuric acid is mainly through benzylmethylcarbinol rather than ethylphenylcarbinol. Another possible route of degradation is by ω-oxidation to β-phenylpropionic acid, which can be subsequently β-oxidized to benzoic acid. According to Raper & Wayne (1928), in the dog 77 % of the administered β-phenylpropionic acid (122 mg./kg.) is excreted as hippuric acid. With doses of about 500 mg./kg. in the same animal Quick (1928)

Table 2. *Excretion of hippuric acid and phenaceturic acid by rabbits receiving alkylbenzenes and related compounds orally*

Results are quoted as the average for three animals, with range in parentheses; the superior figure indicates the number of experiments if other than three. The excretion of metabolites was complete in 24 hr. after dosing. Phenaceturic acid was detected by paper chromatography and roughly estimated; percentage range in parentheses indicates rough estimate as percentage of dose, + +a large amount, +a minor amount, −absent.

Compound	Formula	Dose (m-mole/kg.)	Hippuric acid excretion (% of dose)	Phenaceturic acid excretion	Glucuronic acid conjugation (% of dose)
Benzoic acid	$Ph.CO_2H$	0·75	79 (76, 81)[2]		
Toluene	$Ph.CH_3$	3·0	74 (63–94)		
Ethylbenzene	$Ph.CH_2.CH_3$	3·0	31 (24–36)	(10–20 %)	32*
2-Phenylethanol	$Ph.CH_2.CH_2.OH$	2·0	3† (3–4)	+ +	7*
(±)-Methylphenylcarbinol	$Ph.CH(OH).CH_3$	2·0	28 (26, 30)[2]	−	50*
Acetophenone	$Ph.CO.CH_3$	2·0	19 (17–21)	−	47*
(±)-Mandelic acid	$Ph.CH(OH).CO_2H$	2·0	0 (0–1)	−	
n-Propylbenzene	$Ph.CH_2.CH_2.CH_3$	3·0	15 (13–16)	−	47 (39–56)
β-Phenylpropionic acid	$Ph.CH_2.CH_2.CO_2H$	2·0	47 (41–57)[5]	−	
Cinnamic acid	$Ph.CH{:}CH.CO_2H$	2·0	74 (71–77)	−	
(±)-Ethylphenylcarbinol	$Ph.CH(OH).CH_2.CH_3$	2·0	6 (6–7)	−	54*
Ethyl phenyl ketone	$Ph.CO.CH_2.CH_3$	2·0	8 (7–11)	−	
(±)-Benzylmethylcarbinol	$Ph.CH_2.CH(OH).CH_3$	2·0	19 (17–21)	−	43*
Benzyl methyl ketone	$Ph.CH_2.CO.CH_3$	2·0	20 (14–24)	−	
n-Butylbenzene	$Ph.CH_2.CH_2.CH_2.CH_3$	3·0	2† (0–4)	(15–25 %)	51 (42–60)[2]
γ-Phenylbutyric acid	$Ph.CH_2.CH_2.CH_2.CO_2H$	2·0	3† (2–4)	(80–90 %)	
(±)-Phenylpropylcarbinol	$Ph.CH(OH).CH_2.CH_2.CH_3$	2·0	0 (0–1)	−	72 (66–77)
Phenyl propyl ketone	$Ph.CO.CH_2.CH_2.CH_3$	2·0	4 (2–5)	−	
(±)-Benzylethylcarbinol	$Ph.CH_2.CH(OH).CH_2.CH_3$	2·0	1 (0–2)	−	64 (58–68)
Benzyl ethyl ketone	$Ph.CH_2.CO.CH_2.CH_3$	2·0	4 (3–5)	−	
(±)-Methyl 2-phenylethylcarbinol	$Ph.CH_2.CH_2.CH(OH).CH_3$	2·0	0 (0–0)	(15–20 %)	68 (64–71)
Methyl 2-phenylethyl ketone	$Ph.CH_2.CH_2.CO.CH_3$	2·0	6 (5–7)	+	

* These figures are for doses of 4 m-moles/kg. quoted from Smith *et al.* (1954*a*, *b*, *c*).
† The significance of these values is discussed in the text.

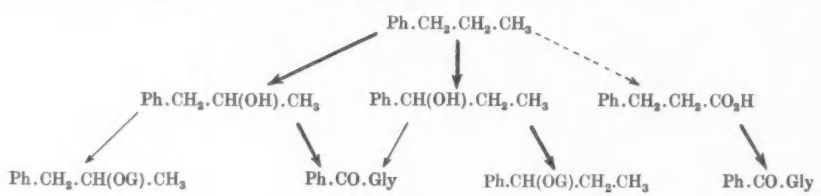

Fig. 2

reported the hippuric acid excretion to be only 28 % of the dose. In rabbits at 300 mg./kg., Table 2 shows the hippuric acid formation to be about 50 % of the dose. With cinnamic acid, the hippuric acid output in the rabbit is 74 % of the dose (Table 2) and is the same as that reported by Raper & Wayne (1928) for the dog.

Cinnamoylglycine has been reported as a trace metabolite in dogs of β-phenylpropionic acid, cinnamic acid and β-hydroxy-β-phenylpropionic acid (cf. Dakin, 1911 and earlier papers). We have shown that this conjugate can be readily detected by paper chromatography. If cinnamoylglycine is a trace metabolite of β-phenylpropionic acid and the latter is an intermediate in propylbenzene metabolism, then cinnamoylglycine could be a trace metabolite of propylbenzene. This glycine conjugate was not detected as a metabolite of propylbenzene, β-phenylpropionic acid or cinnamic acid in the rabbit.

Metabolism of n-*butylbenzene.* The main metabolites of this compound appear to be phenylpropyl- and methylphenethyl-carbinylglucuronides, and phenaceturic acid, and a consideration of all our observations suggests the scheme shown in Fig. 3 for its metabolism. The formation of γ-phenylbutyric acid has not been proved, but we consider it to be a possible reaction, since ω-oxidation of alkyl chains is a known reaction and γ-phenylbutyric acid is known to be oxidized to phenaceturic acid (Raper & Wayne, 1928). Both phenylpropyl- and methyl-2-phenylethyl-carbinols are formed, but only the latter is converted into phenaceturic acid. The third possible carbinol, benzylethylcarbinol, was not detected in the urine and when fed it was not a precursor of phenaceturic acid but was largely excreted conjugated with glucuronic acid (see Table 2). It is probably not a metabolite of butylbenzene. The routes of degradation of the side chain of butylbenzene are therefore by (ω − 1)-oxidation and probably by ω-oxidation. α-Oxidation does take place but does not lead to phenylacetic acid.

It is to be noted in Table 2 that phenyl propyl, benzyl ethyl, and methyl 2-phenylethyl ketones give rise to a small but probably significant (as shown by paper chromatography) hippuric acid output (about 4 %) which is not observed with the corresponding carbinols; otherwise, these ketones appear to be mainly converted into the glucuronides of the corresponding carbinols (see Smith *et al.* 1954*c*). It is thus possible that these ketones undergo a minor metabolic reaction leading to benzoic acid. Loss of the alkyl residue in phenyl propyl and methyl 2-phenylethyl ketones could lead to benzoic acid thus:

$$Ph.CO\vdots CH_2.CH_2.CH_3 \rightarrow Ph.CO_2H$$

$$Ph.CH_2.CH_2.CO\vdots CH_3 \rightarrow Ph.CH_2.CH_2.CO_2H \rightarrow Ph.CO_2H.$$

With benzyl ethyl ketone, loss of the alkyl residue would yield phenylacetic acid:

$$Ph.CH_2.CO.CH_2.CH_3 \rightarrow Ph.CH_2.CO_2H.$$

However, it is to be noted from Table 2 that 2-phenylethanol, which is mainly metabolized to phenylacetic acid, also gives rise to a small apparent hippuric acid output (3–4 %).

Penultimate versus terminal oxidation. Since it has been shown that hippuric acid could arise from propylbenzene by ω- or (ω − 1)-oxidation and phenaceturic acid could arise similarly from butylbenzene, it is pertinent to inquire whether one or both modes of oxidation result in the degradation of these two alkyl chains. Although α-oxidation is important in the degradation of ethylbenzene to hippuric acid, it is not important in the propyl and butyl derivatives, since ethylphenylcarbinol yields little hippuric acid and phenylpropylcarbinol no phenaceturic acid *in vivo*. In any case, α-oxidation is probably a special reaction of alkylbenzenes due to the presence of the benzene ring. ω-Oxidation is a known reaction of alkyl chains of six or more carbon atoms and numerous examples are available, especially in the fatty acid series (see Weitzel, 1951). (ω − 1)-Oxidation of the butyl side chain to form a 3-hydroxybutyl chain has been shown to occur in 5-butyl-5-ethylbarbituric acid (butobarbitone) in the dog (Maynert, 1952) and in 4-butyl-3:5-dioxo-1:2-diphenylpyrazolidine (butazolidine) in man (Burns *et al.* 1953, 1955). The experiments of Titus & Weiss (1955) on the fate of [^{14}C]-5-ethyl-5-(1-methylbutyl)barbituric acid (pentobarbi-

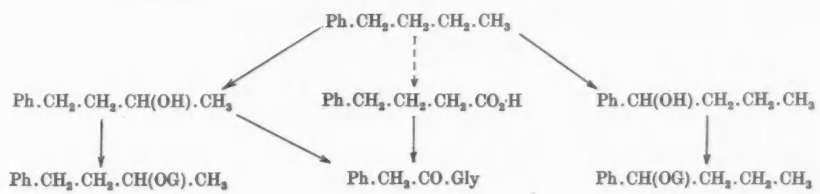

Fig. 3

tone) in the dog suggest that the butyl chain undergoes very little ω-oxidation, since 75·6 % of the dose was excreted as the $(\omega-1)$-oxidation product, 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid, and only 4·5 % as the ω-oxidation product, 5-ethyl-5-(3-carboxy-1-methylpropyl)barbituric acid.

Our present results, however, do not permit a decision as to whether ω- or $(\omega-1)$-oxidation is the more important in the degradation of propyl- and butyl-benzenes. It is significant, however, that the degradation of the $(\omega-1)$-carbinols is of the same order as that of the corresponding alkylbenzenes.

SUMMARY

1. A study has been made of the fate of ethylbenzene, *n*-propylbenzene and *n*-butylbenzene in the rabbit.

2. About 60–70 % of ethylbenzene undergoes α-oxidation to yield methylphenylcarbinol glucuronide and hippuric acid, and 10–20 % undergoes ω-oxidation to yield phenaceturic acid.

3. About 50 % of propylbenzene is excreted as the glucuronides of ethylphenylcarbinol and benzylmethylcarbinol, and about 15 % as hippuric acid. The main precursor of hippuric acid appears to be benzylmethylcarbinol.

4. About 50 % of butylbenzene is excreted as the glucuronides of methyl-2-phenylethylcarbinol and phenylpropylcarbinol, and about 20 % as phenaceturic acid. The precursor of phenaceturic acid appears to be methyl-2-phenylethylcarbinol; phenylpropylcarbinol is not a precursor. Derivatives of benzylethylcarbinol were not detected as metabolites of butylbenzene, and this carbinol is not a precursor of phenaceturic acid.

5. The three alkylbenzenes have been proved to undergo α-oxidation; propyl- and butyl-benzenes also undergo $(\omega-1)$-oxidation of the alkyl side chain. No direct evidence for the ω-oxidation of propyl- and butyl-benzenes has been obtained, but its occurrence cannot be ruled out.

6. A method modified from Gaffney *et al.* (1954) has been described for the determination of hippuric acid in urine.

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Maleic Anhydride in the Study of Naturally Occurring Isomers of Vitamin A

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During the past ten years, several *cis* isomers of vitamin A have been described (Robeson & Baxter, 1945, 1947; Graham, van Dorp & Arens, 1949; Dieterle & Robeson, 1954; Wald, Brown, Hubbard & Oroshnik, 1955; Robeson *et al.* 1955*b*). Their formulae are shown in Fig. 1. It is now known that some of these compounds occur naturaliy. Their presence can be detected by a comparison of chemical and biological assays of vitamin A extracts (Fisher, Kon & Thompson, 1952; Robeson *et al.* 1955*b*), this test being based on the finding that the *cis* isomers so far investigated have lower

CORRIGENDA

Maleic anhydride in the study of naturally occurring isomers of vitamin A

By P. A. Plack

Volume 64 (1956)

p. 56, col. 2, lines 4–5:

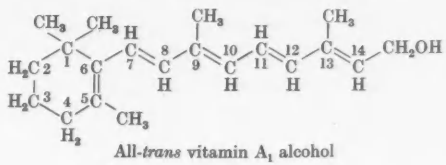
for Robeson *et al.* 1955*b* *read* Ames, Swanson & Harris, 1955

p. 58, col. 2, line 21:

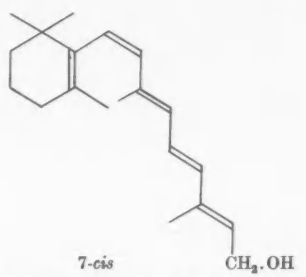
delete (w/v)

biological activities than all-*trans* vitamin A (Harris, Ames & Brinkman, 1951; Ames, Swanson & Harris, 1955), whereas in the Carr–Price reaction their molar extinction coefficients are approximately equal (C. D. Robeson, personal communication to Dr S. K. Kon). Methods for the isolation, characterization and estimation of *cis* isomers are limited. Wald and co-workers (Hubbard & Wald, 1952–53; Wald & Burg, 1955) estimate certain of the isomers by conversion of the alcohol into the aldehyde form, and combination with opsin. With chromatography on calcium phosphate, Bro-Rasmussen, Hjarde & Porotnikoff (1955) have separated quantitatively 13-*cis* vitamin A (neo-vitamin A) from all-*trans* vitamin A_1 and vitamin A_2. Robeson & Baxter (1947) obtained crystalline 13-*cis* vitamin A from fish-liver oil by molecular distillation, fractional chromatography and crystallization. The same isomer was prepared by Chatain & Debodard (1951) by the reaction with maleic anhydride, followed by chromatography and crystallization, and by Dalvi & Morton (1952), who used fractional chromatography only.

At present, the simplest quantitative method of measurement of a *cis* isomer is that of Robeson & Baxter (1947), which estimates 13-*cis* vitamin A in the presence of the all-*trans* isomer by a procedure involving reaction with maleic anhydride. This

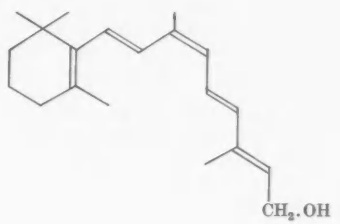


All-*trans* vitamin A_1 alcohol



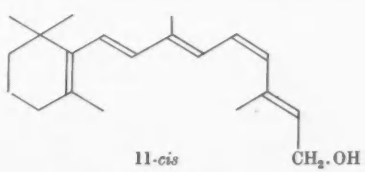
7-*cis*

(Wald, Brown, Hubbard & Oroshnik, 1955)



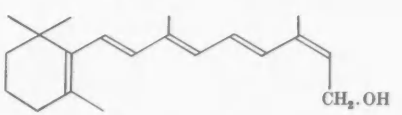
9-*cis*

(Graham, van Dorp & Arens, 1949; Robeson *et al.* 1955 *b*)



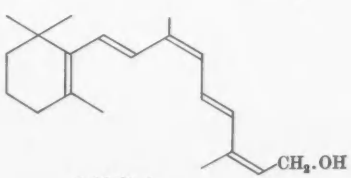
11-*cis*

(Wald *et al.* 1955)



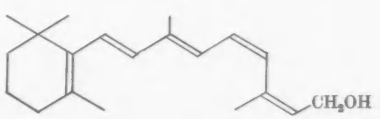
13-*cis*

(Robeson & Baxter, 1947)



9:13-di-*cis*

(Robeson *et al.* 1955 *b*)



11:13-di-*cis*

(Robeson *et al.* 1955 *a*)

Fig. 1. *Cis–trans* isomers of vitamin A alcohol described in the literature, numbered according to the system of Karrer.

method has been applied by Meunier & Jouanneteau (1948), Hayes & Petitpierre (1952) and other workers. Dalvi & Morton (1952) used a similar method, but at a lower temperature. The method described here is an extension of this work.

The combination of maleic anhydride with dienes is well known in organic synthesis. The compound formed by this substance with vitamin A was first investigated by Kawakami (1935) and later by Robeson *et al.* (1955*b*). The latter authors have also given data on the rates of reaction of certain isomers, additional to those of Robeson & Baxter (1947).

In their work on the vitamin A of marine Crustacea, Fisher *et al.* (1952) found a discrepancy between chemical and biological assays on extracts from euphausiids, indicating that *cis* isomers were present. A comparison of the u.v.-absorption curves of an extract from *Meganyctiphanes norvegica* (M. Sars) and of all-*trans* vitamin A alcohol showed that 13-*cis* vitamin A was not one of these (Kon, 1954). A method of estimating other *cis* isomers was therefore required, and the reaction of crystalline all-*trans* vitamin A acetate, palmitate and alcohol, and of vitamin A extracts from natural sources, with maleic anhydride was studied. From it, a method for estimating certain *cis* isomers was evolved, and also a method for their isolation. Results obtained with vitamin A extracts of *M. norvegica*, *Thysanoessa raschii* (M. Sars) and *Pandalus bonnieri* (Caullery) have been used here to illustrate one of these methods, but an account of the isomers of vitamin A present in these and other marine Crustacea will be reserved for a future paper.

NOMENCLATURE

In this paper, vitamin A is used as a general term to cover all forms and isomers of vitamin A_1 when the particular form or configuration does not affect the issue. When reference to a particular isomer is made, the Karrer numbering given in Fig. 1 is used. Weights of vitamin A are given in terms of micrograms of the equivalent quantity of all-*trans* vitamin A alcohol, and were determined either by direct weighing, or by the Carr–Price reaction.

The terms 'fast'- and 'slow'-reacting isomers of vitamin A alcohol refer to those isomers which react with maleic anhydride at a rate comparable respectively, to that of all-*trans* vitamin A alcohol, or of 13-*cis* vitamin A alcohol (Robeson *et al.* 1955*b*).

EXPERIMENTAL

Materials

Crystalline all-*trans* vitamin A alcohol, acetate and palmitate, and α-tocopherol, were obtained from Roche Products Ltd. Light petroleum, b.p. 40–60°, and benzene, pure, crystallizable, from May and Baker Ltd. were redistilled before use. Maleic anhydride (May and Baker Ltd.) was recrystallized at least twice, from either benzene or chloroform, and dissolved in benzene to give a 10% (w/v) solution. Other chemicals were of A.R. quality, or the purest available. The commercial vitamin A concentrate from mixed fish-liver oils was obtained from Crookes Laboratories Ltd., and whale-liver oil was a gift from Hector Whaling Ltd.

Natural sources of vitamin A used were the euphausiid Crustacea *M. norvegica* and *T. raschii*, caught in Loch Fyne, and the decapod crustacean *P. bonnieri*, obtained from the river Clyde.

Methods

General. Previous work from this laboratory (Fisher *et al.* 1952) has shown that in *M. norvegica* and *T. raschii* the major part of the vitamin A is contained in the eyes. The eyes of *M. norvegica* and *P. bonnieri* were removed for use, but with *T. raschii*, owing to its small size, whole animals were extracted. The method of extraction was that given by Thompson, Ganguly & Kon (1949). Saponification was carried out by adding to each 2·5 g. of fat (or less) 5 ml. of ethanol and 1·1 ml. of 60% (w/v) aqueous KOH, and boiling gently for 3 min., adding 20 ml. of water and extracting with 20, 15 and 15 ml. of diethyl ether in a separating funnel. The combined extracts were washed with three lots of 20 ml. of water, dried over anhydrous Na_2SO_4, evaporated to dryness and taken up in a few millilitres of *n*-hexane. The vitamin A in the alcohol form thus obtained was purified by the chromatographic procedure of Thompson *et al.* (1949), except that the column was made up from a slurry of alumina in 8% (v/v) ethanol in *n*-hexane. The whale-liver oil and Crookes vitamin A concentrate were saponified directly and chromatographed as described above.

Ultraviolet-absorption curves were read on a Beckman model DU quartz spectrophotometer with 1 cm. cells, and *n*-hexane as solvent.

Carr–Price reaction. The spectrophotometer of Thompson (1949) was used to measure the intensity of the blue colour produced in this reaction at its absorption maximum, 618 mμ. Workers at Distillation Products Industries have found that the *p*-phenylazobenzoate esters of three *cis* isomers of vitamin A have the same absorption maxima (622 mμ.) and essentially the same $E^{1\%}_{1\,cm.}$ values as this ester of all-*trans* vitamin A (C. D. Robeson, personal communication to Dr S. K. Kon). It is therefore assumed that the alcohol isomers give $E^{1\%}_{1\,cm.}$ values similar to that of all-*trans* vitamin A alcohol. The different absorption maximum found by the American workers is probably instrumental in origin. The method used was as described by Thompson (1949), except that the sample was in either benzene or $CHCl_3$ solution, and 1 drop of acetic anhydride was always used. With vitamin A in benzene solution, the 'stable' reading was very transient, and occasionally it was necessary to use the maximum reading attained. The presence of maleic anhydride tended to stabilize the readings but had no effect on their value. Vitamin A that had combined with maleic anhydride did not give a blue colour in the Carr–Price test.

The calibration of the instrument has been described by Fisher, Kon & Thompson (1956). In the work which follows, instrument readings are given. These may be converted into μg. of vitamin A/ml. of reaction mixture by dividing the readings by 50.

Reaction of vitamin A with maleic anhydride. A solution of the appropriate vitamin A in benzene was prepared at a

concentration of 10–20 μg./ml. A 5 ml. sample was pipetted into an amber-coloured 125 ml. conical flask in a water bath at 25°. To it was added 5 ml. of the 10% solution of maleic anhydride in benzene, previously warmed to 25°. A 1 ml. sample of the mixture was taken immediately, diluted with an equal volume of benzene, and the vitamin A present determined by the Carr–Price test. Subsequent samples were taken at intervals of 0·5, 1, 2, 3, 4, 5 and 6 hr., and diluted as necessary before the Carr–Price test. The flasks were kept corked between the taking of samples. A control flask, containing equal volumes of vitamin A solution and benzene, was always set up, and samples and readings were taken at 0 and 6 hr. The control readings were constant within the experimental error of the Carr–Price test, which is of the order of ±5%.

By beginning with the control flasks, and staggering the initial readings at 5 min. intervals, it was possible to deal with six experimental flasks and six controls in one day.

Determination of 'fast'- and 'slow'-reacting components in a mixture of vitamin A isomers. When the test just described was carried out on saponified vitamin A mixtures from natural sources, and the $\log_{10}$ reading plotted against time, curves such as those in Fig. 2 were obtained. During the first 2 hr., the fall in vitamin A concentration was due mainly to the removal of the 'fast'-reacting components by combination with maleic anhydride. At the end of this time, only 1% of the original 'fast' components should have remained (see results for all-*trans* vitamin A alcohol), and from 2 to 6 hr. the fall will have been due to the reaction of the 'slow' components. These values for the period 2–6 hr. lie on a straight line, and if this line is produced backwards to meet the axis, the intercept gives the $\log_{10}$ value of that part of the initial reading due to the 'slow' components.

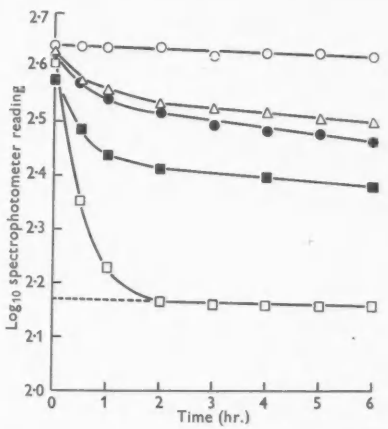


Fig. 2. Reaction with maleic anhydride (5%, w/v) of 13-*cis* vitamin A alcohol (○), and of saponified extracts of vitamin A from whale-liver oil (□), *T. raschii* (△), *M. norvegica* (●) and *P. bonnieri* (■) at concentrations between 7 and 9 μg./ml., in benzene at 25°. The reaction was followed by measurements of the absorption at 618 mμ. in the Carr–Price test due to the unreacted vitamin A (see Methods section).

For whale-liver oil vitamin A, the intercept (see Fig. 2) is 2·170. The initial value was 2·606. Then

$$\text{Percentage of 'slow' components} = \frac{\text{Initial reading due to slow components} \times 100}{\text{Initial reading}},$$

or,

$$\log_{10}(\text{percentage of 'slow' components}) = (2{\cdot}170 + 2) - 2{\cdot}606 = 1{\cdot}564.$$

Hence, percentage of 'slow' components was 37, and percentage of 'fast' components was 63, by difference.

Isolation of the 'slow' components. A solution of the saponified vitamin A extract was prepared in benzene at a maximum concn. of 10 mg./ml. An equal volume of the 10% maleic anhydride solution was added, and the whole incubated for 3 hr. at 25° in a water bath. At the end of this time, some 99% of the 'fast' components would have combined with maleic anhydride. A volume of 2 N-KOH in ethanol equal to that of the mixture was then added, to decompose the excess maleic anhydride and to render the maleic anhydride–vitamin A adducts water-soluble, followed 5 min. later by the same volume of water. The mixture was extracted in a separating funnel with three portions of light petroleum, each equal in volume to five times that of the mixture, and the combined extracts were washed three times with an equal volume of water and dried over anhydrous Na_2SO_4. The light petroleum was removed and a small volume of *n*-hexane added. The solution was then usually chromatographed on weakened alumina by the method of Thompson *et al.* (1949).

Preparation of 13-cis *vitamin A alcohol.* This isomer was prepared from the saponified vitamin A extract from the Crookes vitamin A concentrate, which assayed at 36% of 'slow'-reacting isomers. The method described above was followed, with an initial vitamin A concentration of 3 mg./ml. in benzene. Chromatography was carried out on a column of weakened alumina (20 cm. × 1·8 cm.). The elution of vitamin A by 8% (v/v) ethanol in *n*-hexane was followed by observing its fluorescence with an ultraviolet lamp, and 2 ml. fractions were collected when the vitamin A band was 2 cm. above the base of the column. The first three fractions gave absorption curves in *n*-hexane with maxima at 328 mμ.; they were combined and taken to consist of 13-*cis* vitamin A alcohol.

The biological assay of this preparation was carried out on rats by the vaginal-smear method of Pugsley, Wills & Crandall (1944). Solutions of the 13-*cis* vitamin A alcohol and of crystalline all-*trans* vitamin A acetate were made in arachis oil containing 0·1% α-tocopherol. The vitamin A concentration in each was determined chemically by the Carr–Price reaction, a suitable weight of the oil being dissolved directly in $CHCl_3$ for the test.

RESULTS

Reaction of pure vitamin A isomers with maleic anhydride

The method described for studying the reaction of vitamin A with maleic anhydride gives a series of readings corresponding to the amount of remaining vitamin A that had not reacted with maleic anhydride. For the pure isomers tested, a plot of these readings against time gave an exponential

curve, and a plot of $\log_{10}$ readings against time gave a straight line. Fig. 3 shows this plot for all-*trans* vitamin A acetate. All-*trans* vitamin A palmitate gave an identical straight-line relationship.

All-*trans* vitamin A alcohol (Fig. 3) yielded a similar result but the rate of reaction was much greater. After 1 hr. only 11% of the initial vitamin A was present as such, as against 75% for the esters, and after 3 hr. about 1% was found. From the curve it would be expected theoretically that only 0·13% of the original vitamin A would be present at this time. This discrepancy was due partly to the presence of small quantities of 13-*cis* vitamin A alcohol, possibly produced by isomerization.

13-*cis* Vitamin A alcohol, prepared as described above, reacts very slowly with maleic anhydride, but again the values gave a straight line in a semi-logarithmic plot (Fig. 2).

The straight-line relationship for the pure isomers, between $\log_{10}$ readings and time, indicates that their reaction with maleic anhydride is pseudo-unimolecular, which is to be expected, since on a molar basis the initial concentration of maleic anhydride is some 14000 times that of vitamin A.

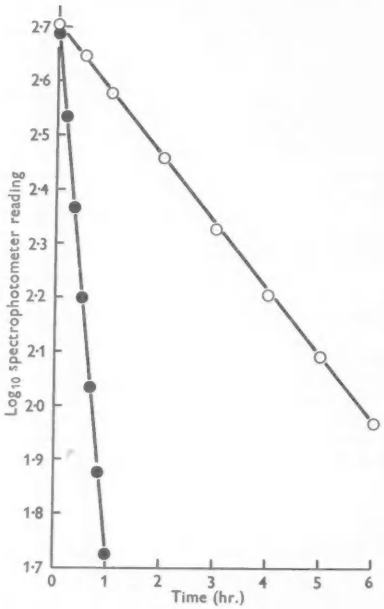


Fig. 3. Reaction of crystalline all-*trans* vitamin A acetate (○), and of crystalline all-*trans* vitamin A alcohol (●) at concentrations of about 10 μg./ml. with maleic anhydride (5%, w/v) in benzene at 25°. The reaction was followed as in Fig. 2.

The velocity constant for the reaction may therefore be determined by the relationship $k = (1/t_{\frac{1}{2}}) \ln 2$, where k is the velocity constant of the reaction and $t_{\frac{1}{2}}$ is the time taken for the concentration of vitamin A to be reduced by one-half.

Under the standard conditions used [25° and 5% (w/v) of maleic anhydride], the following values were found: all-*trans* vitamin A acetate, k 0·280 hr.$^{-1}$; all-*trans* vitamin A alcohol, k 2·24 hr.$^{-1}$; 13-*cis* vitamin A alcohol, k 0·0081 hr.$^{-1}$.

These values are independent of the concentration of vitamin A, provided that 5% (w/v) maleic anhydride constitutes an excess, but dependent on the concentration of maleic anhydride. Fig. 4 shows this latter relationship, and indicates that the primary reaction determining the rate is bimolecular.

Determination of 'fast'- and 'slow'-reacting components

This method makes use of the difference in velocity constants of these two groups of isomers, illustrated by the figures for all-*trans* vitamin A alcohol and 13-*cis* vitamin A alcohol given above. As a test of the method, mixtures of saponified vitamin A extract from *M. norvegica* and all-*trans* vitamin A alcohol were made up as shown in Table 1. The curves obtained are shown in Fig. 5, and from these the percentages of 'fast' components were calculated (Table 1). The calculated values take into account the presence of 'fast' components in the vitamin A of *M. norvegica*.

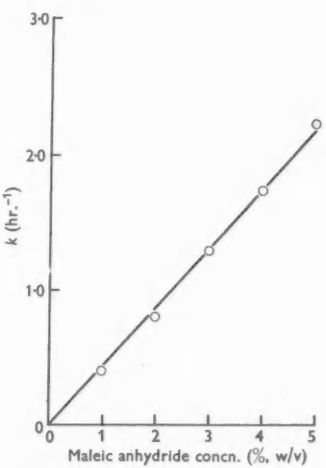


Fig. 4. The reaction of crystalline all-*trans* vitamin A alcohol (9·2 μg./ml.) with various concentrations of maleic anhydride in benzene at 25°. Plot of velocity constants against the concentrations of maleic anhydride.

The reproducibility of the method was tested by repeated determinations of the percentage of 'fast'-reacting components in an *n*-hexane solution of vitamin A from whale-liver oil over a period of 1 month. This solution was stored in the dark at 0°. The values obtained were 62, 61, 64, 64, 64, 64, 62 and 63% of 'fast' components. The results for saponified vitamin A extracts from *M. norvegica*, *T. raschii*, *P. bonnieri* and whale-liver oil, derived from the curves in Fig. 2, are given in Table 2.

Isolation of the 'slow' components

As a test of this method, all-*trans* vitamin A alcohol was allowed to react with maleic anhydride for 30 min. and the remaining free vitamin A (about 32% of the initial) extracted. The u.v.-absorption curve of this recovered material was almost identical with that of the original all-*trans* vitamin A alcohol solution. It follows that all-*trans* vitamin A alcohol that has not reacted with maleic anhydride can be recovered substantially unchanged.

The preparation of 13-*cis* vitamin A alcohol described above gave an u.v.-absorption curve with a maximum at 328 mμ. By biological assay it had 0·68 times the activity of all-*trans* vitamin A alcohol, with true fiducial limits of 81–121% ($P=0·95$). Ames *et al.* (1955) give a mean value of 0·75 times. Application of the maleic anhydride reaction (Fig. 2) showed that the amount of 'fast' components present was too small to estimate.

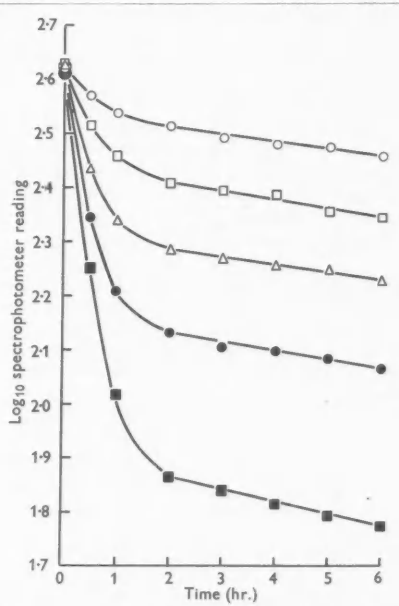


Fig. 5. Reaction of mixtures of a saponified extract of vitamin A from *M. norvegica* and all-*trans* vitamin A alcohol with maleic anhydride in benzene at 25°. ○, 100% *M. norvegica*; □, 80% *M. norvegica* + 20% all-*trans*; △, 60% *M. norvegica* + 40% all-*trans*; ●, 40% *M. norvegica* + 60% all-*trans*; ■, 20% *M. norvegica* + 80% all-*trans*. The reaction was followed as in Fig. 2.

DISCUSSION

Kawakami (1935) suggested that the adduct of vitamin A acetate and maleic anhydride involved the combination of two molecules of the latter with the four double bonds of the vitamin A side chain. Robeson *et al.* (1955*b*), however, found that only the two terminal double bonds of the side chain reacted with one molecule of maleic anhydride, and the present work, which shows that the first and rate-determining reaction is bimolecular, supports their view.

According to Robeson *et al.* (1955*b*), an isomer with *trans* configurations at double bonds 11 and 13 reacts rapidly with maleic anhydride, whereas if one or both are *cis* the rate of reaction is slow. From Fig. 1 it will be seen that the all-*trans*, 7-*cis* (Wald *et al.* 1955) and 9-*cis* (Graham *et al.* 1949; Robeson *et al.* 1955*b*) isomers should comprise the 'fast'-reacting group, and the 11-*cis* (Wald *et al.* 1955),

Table 1. *Percentage of 'fast' components in mixtures of a saponified vitamin A extract from* M. norvegica *and all-*trans *vitamin A alcohol*

Solutions of saponified vitamin A extract from *M. norvegica* and of all-*trans* vitamin A alcohol in benzene, of the same strength by the Carr–Price reaction, were mixed in the proportions shown.

Percentage composition of mixture		Percentage of 'fast' components	
M. norvegica vitamin A	All-*trans* vitamin A	Calculated*	Found
100	0	—	19
80	20	35	35
60	40	51	51
40	60	68	64
20	80	84	80

* In the calculation of the percentage of 'fast' components, allowance has been made for the presence of these in the extract from *M. norvegica*.

Table 2. *Percentage of 'fast' and 'slow' components in vitamin A from marine sources*

Estimations were made as in the Methods section.

Source	Fast	Slow
Thysanoessa raschii, whole animals	17	83
Meganyctiphanes norvegica, eyes	19	81
Pandalus bonnieri, eyes	29	71
Whale-liver oil	63	37

13-*cis* (Robeson & Baxter, 1947), 9:13-di-*cis* (Robeson *et al.* 1955*b*) and 11:13-di-*cis* (Dieterle & Robeson, 1954; Robeson, Blum, Dieterle, Cawley & Baxter, 1955*a*) isomers the 'slow'-reacting group. There is one possible exception to this rule, namely 7-*cis* vitamin A. Wald *et al.* (1955) ascribe this formula to neovitamin A*b*, which is 'slow'-reacting (Robeson *et al.* 1955*a*), but the latter authors suggest the 11:13-di-*cis* structure.* The relative amounts of these two groups may be determined by the method described.

The test of Robeson & Baxter (1947) does not allow for the possible presence of 'slow'-reacting isomers other than 13-*cis*, but these do occur naturally. Neovitamin A*b* has been found in the eyes of lobsters (Wald & Burg, 1955); Fisher, Henry, Kon, Plack & Thompson (1955) found in *M. norvegica* small amounts of an isomer which resembles the 11-*cis* compound,* and Lambertsen & Braekkan (1955) have suggested that 9:13-di-*cis* vitamin A may be present in *Pandalus borealis*.

No account has been taken of the possible presence of vitamin A_2, which has been found, apart from fresh-water fish, in marine fish-liver oils (Edisbury, Morton, Simpkins & Lovern, 1938; Gillam, Heilbron, Jones & Lederer, 1938; Lovern, Morton & Ireland, 1939; Wald, 1938–39; Cama *et al.* 1952; Cama & Morton, 1953; Bro-Rasmussen *et al.* 1955). On theoretical grounds, it seems likely that the *cis–trans* isomers of this vitamin will react in the same way as those of vitamin A_1, but samples of definite configuration have not been available for testing.

The difference in the rates of reaction with maleic anhydride of all-*trans* vitamin A alcohol on the one hand and of all-*trans* vitamin A acetate and plamitate on the other is very marked. The behaviours of the alcohol and ester forms in adsorption chromatography are also markedly different, and the two effects are possibly due to dissimilar electron distributions of the alcohol and esters. The 'slow'-reacting isomers of vitamin A alcohol also have different rates of reaction. In Fig. 2 it will be seen that over the period 2–6 hr. the slopes of the curves for 13-*cis* vitamin A and saponified whale-liver oil (of which 13-*cis* is the main 'slow' component) correspond, whereas those for *T. raschii*, *M. norvegica* and *P. bonnieri* are greater, but roughly similar among themselves. These rates of reaction may possibly be due to different rates of isomerization to the 11:13-di-*trans* form, rather than to different rates of combination with maleic anhydride. The method is not sufficiently accurate at the moment to enable individual components to be identified by this property.

The method of separating the 'slow'-reacting isomers from the 'fast'-reacting isomers is relatively simple compared with such processes as molecular distillation (cf. Robeson & Baxter, 1947), and is especially suitable when only small amounts of material are available. If only one 'slow'-reacting isomer is present, this method is capable of giving a high degree of purification, without resort to crystallization, which with small quantities of material is not possible. When more than one 'slow' reactor is present, application of this method provides a less complex mixture than the original for the application of fractional chromatography and other techniques.

SUMMARY

1. The reactions of crystalline vitamin A alcohol, acetate and palmitate with maleic anhydride in benzene solution have been studied. The alcohol form reacts much more rapidly than the esters.

2. A study of the reaction of maleic anhydride with saponified vitamin A extracts from various marine sources enables the percentages of 'fast'- and 'slow'-reacting isomers to be calculated. (The former comprise all-*trans*, 7-*cis* and 9-*cis* vitamin A, and the latter 11-*cis*, 13-*cis*, 9:13-di-*cis* and 11:13-di-*cis* vitamin A.)

3. The 'slow'-reacting isomers may be isolated from the reaction mixture simply by adding ethanolic potassium hydroxide, then water, and extracting with light petroleum. An example is given of the preparation of 13-*cis* vitamin A alcohol from a commercial vitamin A preparation.

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* *Note added* 29 May 1956. According to Oroshnik's latest work [Oroshnik, W. (1956) *J. Amer. chem. Soc.*, **78**, in the press], neovitamin A*b* is 11-*cis* vitamin A and the isomer with $\lambda_{max.}$ 311 mμ. in *n*-hexane (11-*cis* vitamin A of Wald *et al.* 1955) is 11:13-di-*cis* vitamin A.

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The Action of Culture Filtrates of the Fungus *Myrothecium verrucaria* on β-Glucosans

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The soil fungus *Myrothecium verrucaria* (*Metarrhizium glutinosum*) is well known for its ability to decompose cellulose, and it has been used in various investigations of that subject (cf. Siu, 1951). This paper describes some properties of a series of culture filtrates of the fungus and our observations seem interesting from two points of view.

(1) There is at present doubt about the nature of extracellular fungal cellulase. From comparisons of various fungi, including *M. verrucaria*, Reese, Siu & Levinson (1950) and Reese & Levinson (1952) concluded that an enzyme C_1 (cellulase; Siu, 1950) transforms insoluble complex cellulose into single molecules of water-soluble cellulose, and that a second enzyme C_x, a β-1:4-glucosidase, breaks these glucose chains into fragments mainly of two units, i.e. cellobiose. They sometimes found glucose in their reaction mixtures, but did not say clearly how they thought it might be formed. On the other hand Whitaker (1953), working with purified preparations from *M. verrucaria*, concluded that there is only a single enzyme, capable of degrading complex cellulose to glucose. Kooiman, Roelofsen & Sweeris (1953), also working with *M. verrucaria*, agreed with Whitaker save that they demonstrated the presence of a distinct cellobiase. Our work confirms that of Kooiman *et al.*

(2) We have found that *M. verrucaria* filtrates also attack β-1:3-glucosides such as laminarin and laminaribiose and, in addition, the β-glucosan isolated from barley by Preece & Mackenzie (1952). This has been analysed chemically by Aspinall & Telfer (1954), who concluded that it contains unbranched chains of β-D-glucopyranose units with approximately equal proportions of 1:3- and 1:4-linkages. The course of its breakdown by our filtrates confirms this view and leads to suggestions about the relative positions of these 1:3- and 1:4-linkages.

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METHODS

Preparation of the culture filtrate

The strain of *M. verrucaria* used was obtained from the Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands) and was maintained on the mineral salt solution detailed below, to which a strip of sterile filter paper was added.

Roux flasks of 1 l. capacity, containing 850 ml. of an inorganic salt solution (KH_2PO_4, 2·68 g.; K_2HPO_4, 2·09 g.; NH_4NO_3, 3·0 g.; $MgSO_4,7H_2O$, 2·22 g.; distilled water to 1 l.) and 20 g. of cellulose powder (Whatman, standard grade), were sterilized, inoculated from the stock cultures and incubated at 25°. By passing a steady stream of sterile air through the culture, the cellulose powder was kept in suspension and the medium aerated. After 2 weeks, the cultures were filtered and sodium azide (0·1%) was added to the filtrates as preservative. Azide was chosen because it is not volatile. Preliminary experiments showed that the same rate of reaction was obtained when the filtrates were preserved with 0·1% azide, with 0·2% fluoride, or by saturation with toluene or thymol.

The activities of five filtrates (*A–E*) are described in this paper. Protein-nitrogen contents of filtrates *A* and *C* were estimated and were found to be 0·02 and 0·006 mg. of N/ml. respectively. The pH of the filtrates varied only between 6·0 and 6·1; addition of NaN_3 raised the pH to 6·4. The filtrates were stored at 0° until required. No decrease in enzymic activity was found to occur within 2 weeks, nor was any reduction in activity detected after several months when stored at −10° or as an acetone-dried powder prepared by adding 4 vol. of pre-cooled acetone at 0°, and centrifuging and drying.

Heat treatment of the filtrate

One portion of the filtrate was left untreated; a second was heated rapidly over a naked flame, with shaking, to 60°, immersed in a water bath at the same temperature for 10 min. and then cooled rapidly; a third was boiled for 5 min.; and a fourth was boiled for 10 min. The boiled filtrates were used only in viscometric experiments.

Substrates

Cotton wool. Absorbent-cotton wool was boiled for 30 min. with 1% aqueous NaOH to remove residual hemicelluloses, washed and dried. It was found that this material was attacked rather more rapidly than unpurified absorbent-cotton wool, suggesting that slight opening out of the molecule had taken place.

Filter-paper powder. Whatman's cellulose powder for chromatography, standard grade. The degree of polymerization was found to be 160 (by G. van Nederveen, Fibre Research Institute T.N.O., Delft, The Netherlands).

Regenerated cellulose. This was prepared by the method of Hirst, Isherwood, Jermyn & Jones (1949). It is believed to have had a chain length of about 220 anhydroglucose units. The product was not dried but was stored at −5° at a moisture content of 78%; if dried, it rehydrates slowly and is comparatively slowly attacked.

Soluble methylcellulose (I.C.I. Ltd.). This has 0·45–0·5 methyl group/anhydroglucose unit, and its molecule must, presumably, contain a proportion of unsubstituted cellobiose units.

Cellobiose (Thomas Kerfoot and Co. Ltd.). This was chromatographically free from glucose and oligosaccharides.

Laminarin and laminaribiose. These were the respective gifts of Mr F. T. Walker and Dr W. A. P. Black of the Institute of Seaweed Research, Inveresk.

Barley β-glucosan. This polysaccharide was first isolated by Preece & Mackenzie (1952) and was given by Professor I. A. Preece (Heriot-Watt College, Edinburgh).

Reductometric determinations

Reaction mixtures consisted of culture filtrate containing 1% (w/v) of substrate, except with cellobiose, where the concentration varied. In earlier experiments they were incubated, unshaken, at 30° but in later experiments, when shaking was adopted, they were incubated at 37°. In controlled experiments, unshaken, no difference in reaction velocity was observed at the higher temperature. Samples were filtered and total reducing power was determined on portions (diluted if necessary) by the method of Somogyi (1952). It was found that 1 ml. of 5 mN-$Na_2S_2O_3$ solution was equivalent to 0·143 mg. of anhydrous glucose or 0·212 mg. of anhydrous cellobiose. When it was desired to estimate glucose and cellobiose separately, glucose was removed by fermentation with baker's yeast (not fermenting cellobiose) and then calculated as the difference between the initial and residual reducing powers.

Chromatography

All solutions to be chromatographed were de-ionized by successive passage over columns of Zeo-Karb 225 and Deacidite G. They were then evaporated to dryness and the residue was taken up in a volume of 10% *iso*propyl alcohol to give a solution roughly equivalent in reducing power to 2% glucose, or in 1 ml., whichever was the greater. Where the resultant extract fell below the desired concentration, proportionately more spots were put on the papers. All chromatograms were run on Whatman no. 1 paper. The papers were irrigated with ethyl acetate–propan-1-ol–water (57:32:13, by vol., or 57:42:20, by vol., a mixture giving better separation) at 20°, and developed either by spraying with a solution of aniline phthalate in water-saturated butanol and heating or with alkaline $AgNO_3$ by the method of Trevelyan, Proctor & Harrison (1950).

Viscometric determinations

Culture filtrate was added to 0·7% (w/v) aqueous methylcellulose at 25°. Viscosity measurements were made immediately and at suitable intervals by timing the fall of the meniscus between two points on a 5 ml. bulb pipette. All solutions and apparatus were kept overnight at 25° before use.

RESULTS

Cellobiose

The five culture filtrates differed in their action on cellobiose. With three (*A–C*), the cellobiose was hydrolysed almost completely to glucose, an activity which was totally absent from the corresponding part of the filtrate which had been heated for 10 min. at 60°. This was observed both reductometrically (Fig. 1), and chromatographically

(Fig. 2) with both filtrate and acetone-dried powder, and indicates the occurrence of heat-labile cellobiase.

On the other hand, with two filtrates (*D* and *E*) acting on cellobiose, no increase in reducing power could be detected. However, chromatograms of the reaction products of filtrate *E* revealed the presence of three reducing ingredients, one of which ran more slowly than cellobiose and might be cellotriose. This spot did not appear when heated filtrate was used, nor was any glucose produced.

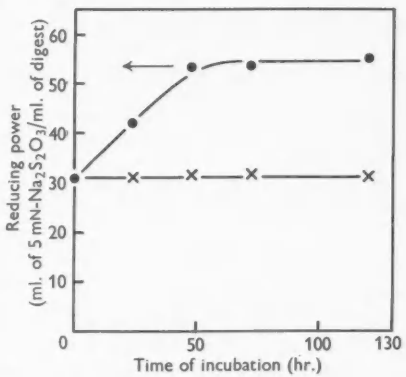

Fig. 1. Hydrolysis of cellobiose by culture filtrate *A* of *M. verrucaria*. Unheated (●) and heated (×) filtrate (30 ml.) acting on cellobiose (200 mg.) at 30° without shaking. Arrow indicates 100% conversion.

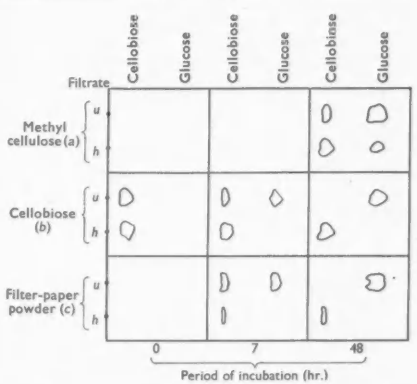

Fig. 2. Chromatograms of products of action of culture filtrate *C* of *M. verrucaria*, unheated (*u*) and heated (*h*), on cellobiose, methylcellulose and filter-paper powder at 30°: *a* with culture filtrate, *b* and *c* with acetone-dried powder (1% solution in 0·05M succinate–borate buffer, pH 5·6). Solvent composition, 57:32:13; sprayed with aniline phthalate (see Methods section).

Soluble methylated cellulose

When culture filtrates were added to a methylcellulose solution, a rapid fall in viscosity ensued. Heating a filtrate (*E*) at 60° for 10 min. reduced the rate of this change by about half (Fig. 3); but it was not completely abolished by boiling for several minutes, as Kooiman *et al.* (1953) also observed. In experiments with another filtrate (*C*) the change was followed virtually to completion. The viscosity approached that of water (specific viscosity $< 1{\cdot}1$), and chromatographic examination of the reaction mixture revealed glucose and traces of cellobiose. Heating the filtrate for 10 min. at 60° had two effects: the reaction was again slowed down but not abolished, and the reaction products contained a higher ratio of cellobiose to glucose (Fig. 2). This difference corresponded with the previously observed inactivation, by such heating, of the system attacking cellobiose. Since, however, some glucose was present (Fig. 2) it must have been produced by the system attacking the cellulose chain. This system, largely heat-stable and apparently capable of degrading cellulose chains at least in large part into cellobiose plus a little glucose, was evidently distinct from the cellobiase in the filtrates.

Later experiments, with different filtrates and better chromatographic technique, gave chromato-

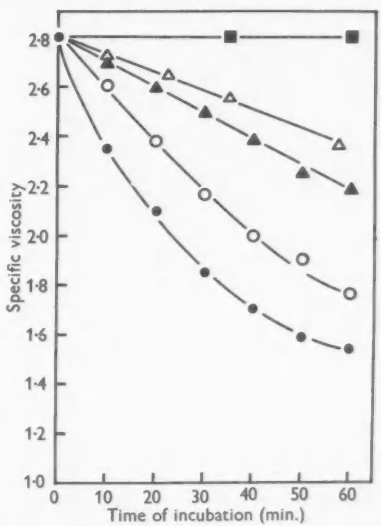

Fig. 3. Diminution of viscosity of methylcellulose solution by culture filtrate *E* of *M. verrucaria*. The digest contained 0·5 ml. of filtrate and 30 ml. of 0·7% (w/v) aqueous methylcellulose at 25°. ●, Unheated filtrate; ○, filtrate heated at 60° for 10 min.; ▲, filtrate boiled for 5 min.; △, filtrate boiled for 10 min.; ■, water (control).

grams in which there were present several slower-running reducing compounds, besides cellobiose. This too was observed by Kooiman *et al.* (1953).

Insoluble cellulose and derivatives

The actions of three different filtrates (*A*, *D* and *E*), and of a solution of the acetone-dried powder of filtrate *C*, on filter-paper powder were followed reductometrically or chromatographically. All the reductometric measurements agreed in showing a rapidly falling rate of breakdown, which practically ceased when only a small fraction of the cellulose had appeared as reducing compounds (Fig. 4). If glucose were the only reducing substance produced, it would correspond to less than 2 % of the original cellulose; if cellobiose, to less than 4 %. The actual percentage breakdown is therefore probably of this order.

In other respects there were differences. With the first filtrate (*A*), 10 min. heating at 60° stopped the production of reducing power almost, but not quite, completely (Fig. 4α). This filtrate contained an active cellobiase (that of Fig. 1). With the acetone-dried powder from a filtrate *C*, chromatograms (Fig. 2) showed the chief product with an unheated solution to be glucose; with the heated solution it was cellobiose, traces of glucose appearing only after incubation for 120 hr. This confirmed the previous indication, with methylcellulose, that glucose can be formed by cellulase alone, but only slowly.

With later filtrates (*D* and *E*) the standard heating did not diminish the production of reducing substances to anything like the same degree. Filtrate *D* (used after 3 months' storage at −10°) showed only about 50 % inactivation (Fig. 4β) and, in an attempt to explain this difference, further experiments were made with the same and with a further filtrate (*E*) in which the reaction mixture was shaken continuously. This considerably accelerated the action of the unheated filtrates and somewhat raised the proportion of filter-paper powder broken down, especially in the last experiment where the shaking was vigorous. But the inactivation by heating was still variable, being roughly three-quarters and two-thirds in the last two experiments (Figs. 4γ, δ). These differences are still unexplained.

The low degrees of conversion and the variable results of heating, in these experiments, raised doubts about the suitability of the substrate. Hence experiments were made with other forms of cellulose: cotton wool, and a regenerated cellulose, prepared as described above. Stored filtrate *D*, mentioned above in Fig. 4γ, was used and the mixtures were shaken at a moderate speed. The reducing powers of the mixtures after different times are given in Fig. 5. With cotton wool, the course of the reaction resembled that for filter-paper powder with the same filtrate in that it slowed down greatly after about 12 hr., though the conversion here slightly exceeded that attained with filter-paper powder (cf. Fig. 4γ). The inactivation as a result of heating appeared greater when the heated filtrate was applied to cotton wool. With the more soluble regenerated cellulose, the reaction was much more rapid and proceeded much further, while the degree of inactivation appeared slightly less than when acting on filter-paper powder.

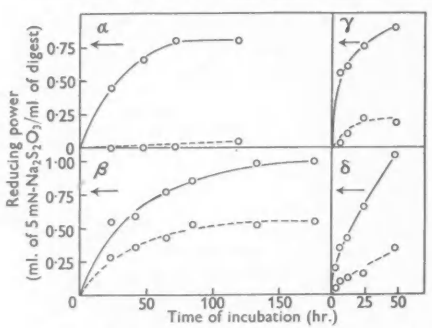

Fig. 4. Action of unheated (—) and heated (- - -) culture filtrates of *M. verrucaria* on filter-paper powder (1 %, w/v) under different conditions. α, Filtrate *A*, 30°, unshaken; β, filtrate *D*, 30°, unshaken; γ, filtrate *D*, 37°, shaken moderately; δ, filtrate *E*, 37°, shaken vigorously. Arrow indicates 1 % conversion (calculated as glucose).

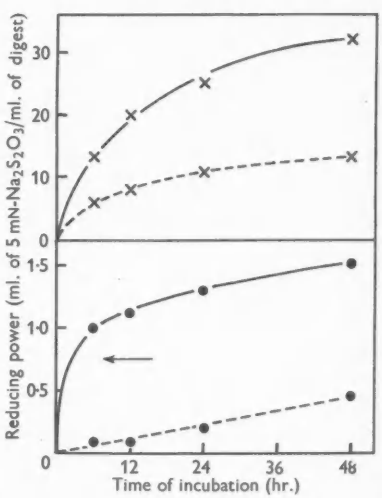

Fig. 5. Action of culture filtrate *D* of *M. verrucaria*, unheated (—) and heated (- - -), on regenerated cellulose (×) and cotton wool (●) at 37° with moderate shaking. Arrow indicates 1 % conversion (calculated as glucose).

Comparison of actions on insoluble cellulose, soluble-cellulose derivatives, and cellobiose

After these experiments we wished to confirm the results by comparing simultaneously the actions of a single filtrate, unheated and heated, on all the various substrates. To accelerate the reaction and avoid premature stasis, the reaction mixtures were shaken vigorously. This filtrate (*E*) had a small reducing power of its own which did not change during incubation; hence this has been deducted from the other determinations which are presented in Fig. 6.

Compared with the filtrate *D* over the first 12 hr., the activity of filtrate *E*, unheated, was about the same on cotton wool, about two-thirds as great on filter-paper powder, and twice as great on regenerated cellulose. Corresponding with the more vigorous shaking, however, the breakdown of cotton wool (like that of filter-paper powder in Fig. 4*δ*) did not slow down noticeably after the first few hours. The inactivation by heating, on cotton wool, filter-paper powder or regenerated cellulose, was less in proportion than with the previous filtrate. The two soluble derivatives were much more rapidly attacked than the insoluble ones, methylcellulose especially; but the breakdown of methylcellulose ceased abruptly at roughly 30% conversion, conceivably because the remaining fragments were too highly methylated to be attacked.

After 53 hr., besides total reducing powers, reducing powers after fermentation of the glucose by baker's yeast were determined. The difference between these values can be ascribed with some confidence to the glucose present, which the yeast ferments; the unfermented residue might include higher oligoses besides cellobiose. These results (Table 1) show clearly that the proportion of cellobiose to glucose was greater with heated than with unheated filtrate. This in itself would account for part of the apparent inactivation on heating shown in Fig. 6. Hence another estimate making allowance for this difference in composition has been calculated in Table 1, though this estimate too is clearly

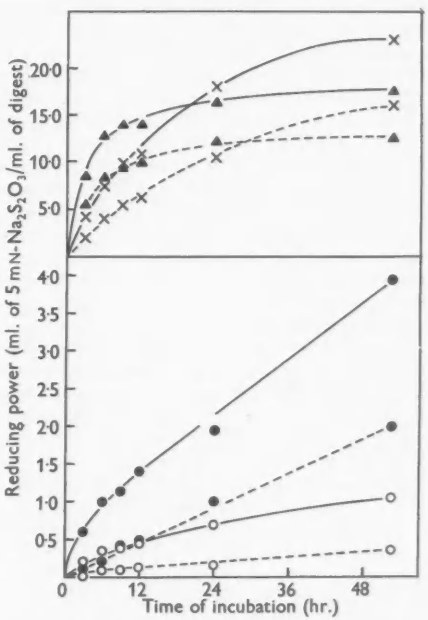

Fig. 6. Action of culture filtrate *E* of *M. verrucaria*, unheated (—) and heated (- - -), on cotton wool (●), filter-paper powder (○), regenerated cellulose (×) and methylcellulose (▲) at 37° with vigorous shaking.

Table 1. *Action of* M. verrucaria *culture filtrate on cellulosic substrates*

Action of filtrate *E*, unheated (*u*) and heated (*h*) at 60° for 10 min., on cotton wool, filter-paper powder, regenerated cellulose, methylcellulose and cellobiose (all 1%, w/v) after 53 hr. at 37°. Glucose and unfermentable sugars (cellobiose, etc.) were determined as in the Methods section. Weights refer to 1 ml. of digest.

Substrate	Filtrate	Glucose (mg.)	Cellobiose, etc. (mg.)	Glucose + cellobiose (mg. of anhydroglucose)	Approx. breakdown of substrate (%)
Cotton wool	*u*	0·33	0·36	0·64	6·4
	h	0·09	0·35	0·42	4·2
Filter-paper powder	*u*	0·12	0·05	0·16	1·6
	h	0·015	0·055	0·065	0·65
Regenerated cellulose	*u*	1·1	3·35	4·2	42
	h	0·35	2·9	3·06	30·6
Methylcellulose	*u*	1·28	1·85	2·9	29
	h	0·32	2·25	2·43	24·3
Cellobiose	*u*	1·45	*	*	*
	h	Nil	12·0	Nil	0

* Incalculable because of production of cellotriose.

approximate, because the influence of oligoses is undetermined and it is unlikely that the proportions of the various constituents were the same throughout.

With all forms of cellulose, relatively small amounts of glucose were produced by the heated filtrate, which was unable to produce any glucose from cellobiose. Hence it is clear here, as in earlier experiments, that this glucose must have been produced by the cellulase system.

In general, the chromatograms confirmed and extended the results of the reductometric experiments. In particular the changes in the proportion of glucose and cellobiose were clearly visible. Methylcellulose, both with heated and unheated filtrate, showed much less cellobiose chromatographically than the reductometric figures in Table 1 indicate. The presence of reducing compounds in addition to cellobiose and glucose was evident in slower-running spots on the chromatogram, conceivably due to higher, possibly methylated, saccharides (see above).

This filtrate was one of those which gave no significant change in reducing power in the presence of cellobiose. The fermentation experiments nevertheless revealed about 1·5 mg. of glucose/ml. of reaction mixture after 53 hr., and both these experiments and the chromatograms revealed greater quantities of cellobiose with the heated (12 mg.) than with the unheated filtrate (< 10 mg.). This difference was clearly not the result simply of cellobiase activity, and with unheated filtrate and cellobiose the chromatogram revealed the existence of a reducing compound running more slowly than cellobiose. Similar observations led Kooiman *et al.* (1953) to suggest a transglycosidation reaction of the type

cellobiose + cellobiose → glucose + cellotriose,

on which basis the production of 1·5 mg. of glucose from an initial 12 mg. of cellobiose/ml. corresponds to the progress of about half the cellobiose through the reaction. This system awaits further investigation, but it is clear that whatever the reaction may be, it was wholly abolished when the filtrate was heated, so that the enzyme involved must be different from that attacking cellulose.

Laminarin and laminaribiose

The filtrate (*D*) used in this and the following reaction lacked cellobiase. Its action, unheated, on laminarin was to produce glucose and laminaribiose, whereas, heated, it produced only very small amounts of glucose and no laminaribiose. Both heated and unheated filtrates produced glucose from laminaribiose, no difference in amount being detected chromatographically. It appears therefore that the activity of the filtrate towards laminarin was largely destroyed by heating at 60° for 10 min., whereas that towards laminaribiose was unaffected. These reactions to heating are the opposite of those of the corresponding steps in cellulose breakdown.

Barley β-glucosan

Unheated-culture filtrate attacked barley β-glucosan with the production of cellobiose, glucose and laminaribiose in roughly equal quantities, as estimated chromatographically. Heated filtrate produced roughly the same amount of cellobiose, less glucose and no laminaribiose. These results, together with those with laminarin and laminaribiose, are summarized in Table 2.

DISCUSSION

The variability in enzyme activity between filtrates of cultures, grown under conditions ostensibly the same, is a prominent feature of the above results. The observed differences in cellobiase activity clearly correspond with the various results recorded by Kooiman *et al.* (1953), by Whitaker (1953) and by Levinson, Mandels & Reese (1951). As all attack on cellobiose was prevented when filtrates were heated

Table 2. *Action of* M. verrucaria *culture filtrate on* β-1:3- *and* β-1:4-*linked polyglucoses*

Action of filtrate *D*, unheated (*u*) and heated (*h*) at 60° for 10 min., on laminarin, laminaribiose, cellobiose and barley β-glucosan for 24 hr. at 30°; −, + and + + represent rough quantitative assessments of the sugars appearing on paper chromatograms prepared as in the Methods section.

		Sugars detected on chromatograms		
Substrate	Filtrate	Glucose	Cellobiose	Laminaribiose
Laminarin	*u*	+ +	−	+
	h	+ (very faint)	−	−
Laminaribiose	*u*	+ +	−	+ +
	h	+ +	−	+ +
Cellobiose	*u*	+	+	−
	h	−	+ +	−
Barley β-glucosan	*u*	+ +	+ +	+ +
	h	+	+ +	−

for 10 min. at 60°, while that on several forms of cellulose was not, it is plain that the cellobiase is distinct from cellulase.

With heated filtrates, glucose was produced as well as cellobiose from several types of cellulose, which confirms the conclusions of Whitaker and of Kooiman *et al.* that cellulase can produce glucose directly. Nevertheless, in filtrates with an active cellobiase, the ratio glucose:cellobiose was much greater with the unheated than with the heated filtrate, so that, in such filtrates most of the glucose must be produced by cellobiase. The activity towards cellulose was roughly halved by the standard heat treatment, though the exact degree of inactivation varied from filtrate to filtrate, and between substrates.

The broadly similar degrees of inactivation on heating, with insoluble or soluble forms of cellulose as substrate, and in both viscometric and reductometric observations on methylcellulose, are consistent with the view that all forms of cellulose are broken down by a single, chain-splitting β-1:4-glucosidase (C_x), which does not attack cellobiose. But this view is unsatisfying in detail. When two different filtrates acted on several different forms of cellulose, there were quite different proportions between the relative rates at which the several substrates were broken down. Whitaker (1953), working with successively purified fractions of a single filtrate, observed that the proportions between attack on various substrates remained constant, and from this inferred that his preparations contained a single enzyme. On that basis, our different experience suggests that the situation in a less purified preparation may be more complex. Further, in one experiment with cellulose powder (Fig. 4α), we observed an inactivation on heating which was so unusually large (95 %) as to hint that some different enzyme might be involved (cf. Saunders, Siu & Genest, 1948). Clearly, however, these experiments give no definite evidence of the existence of a separate enzyme C_1.

With insoluble cellulose, only a small fraction was broken down at an appreciable rate, and even with soluble-cellulose derivatives breakdown was less than 50 %. With regenerated cellulose this might have been due to an inhibitory effect of cellobiose on cellulase (cf. Reese, Gilligan & Norkrans, 1952), but with cotton wool and filter-paper powder the stasis seemed to be more directly related to the solubility of the substrate than to the small amounts of cellobiose present. This, together with the fact that shaking increases the conversion, suggests that cessation of attack on the cellulose complex is caused by some physical obstacle.

There was some evidence, with filtrates lacking cellobiase, for the existence of another enzyme destroying cellobiose with the production of glucose and an oligosaccharide higher than cellobiose. This activity, like that of cellobiase, is destroyed on heating at 60° for 10 min. It is not known whether this enzyme is present in all filtrates, since its action would be masked in the presence of an active cellobiase; nor has it been decided what factors in the culture determine whether this enzyme, or cellobiase, is produced in the medium, or both.

The ability of our filtrates to degrade laminarin has recently been confirmed by Morris (1955), who records the production of readily fermentable reducing substances, presumably glucose. The action of the filtrate in producing laminaribiose and greater amounts of glucose from laminarin is analogous to the production of cellobiose and glucose from cellulose, and to the production of xylobiose and xylose by *A*- and *B*-xylanases (Sørensen, 1952). However, the behaviour of the 1:3-glucosidases after 10 min. heating at 60° is the opposite of that observed with the corresponding 1:4-ases; i.e. the hydrolysis of laminarin is almost completely prevented by such treatment and that of laminaribiose is practically unaffected.

When the filtrates act on the barley β-glucosan, the products are cellobiose, laminaribiose and glucose. The cellobiose could be produced by the β-1:4-glucosidase (cellulase), the laminaribiose by the corresponding 1:3-ase, and the glucose, if in no other way, by the action of laminaribiase on laminaribiose. When the culture filtrate has been heated at 60° for 10 min. before reacting with the β-glucosan under otherwise identical conditions, at least as much cellobiose is produced, less glucose and no laminaribiose.

This might be expected from the observed behaviour of the enzymes. The chain-splitting β-1:3-ase is inactivated, hence no laminaribiose should be produced, while the activity of the laminaribiase remains unimpaired. On the other hand, there is partial inactivation of the chain-splitting 1:4-ase producing cellobiose, while the systems producing glucose from cellobiose would be totally inactivated. Hence the production of glucose by either the 1:3- or the 1:4-route should be negligible. Nevertheless, there is a residual production of glucose, conceivably due to the ability of cellulase to produce glucose directly. The above argument depends on the assumption that the β-1:3- and β-1:4-linkages occur in separate groups in the β-glucosan molecule. If, on the contrary, the 1:3- and 1:4-linkages were to occur alternately in the glucose chains the action of the 1:3-ase would yield cellobiose and not laminaribiose, its inactivation by heating should result in a decreased production of cellobiose and the 1:4-ase would not produce any cellobiose. If the 1:3- and 1:4-linkages alternated in that way, our results would be incomprehensible, hence the view is

favoured that at least the greater proportion of these linkages occur in separate homologous groups in the β-glucosan of barley.

SUMMARY

1. Cell-free culture filtrates of *Myrothecium verrucaria* contain a cellulase, producing cellobiose and glucose from complex and single-chain cellulose molecules, which is partially inactivated by heating at 60° for 10 min.

2. No evidence has appeared of the existence, in these filtrates, of a further enzyme breaking down complex cellulose into straight-chain fragments.

3. They also contain, though not always, cellobiase, hydrolysing cellobiose to glucose, which is completely inactivated in 10 min. at 60°.

4. When cellobiase is absent there is evidence of transglycosidase activity, producing glucose and cellotriose from cellobiose. This activity is also completely abolished by heating for 10 min. at 60°.

5. In addition to β-1:4-glucosidases, culture filtrates of *M. verrucaria* contain β-1:3-glucosidases, hydrolysing laminarin and laminaribiose.

6. When β-1:3- and β-1:4-ases act together on barley β-glucosan the products are cellobiose, laminaribiose and glucose.

7. The results obtained after the filtrate has been heated at 60° for 10 min. suggest that the β-1:3- and β-1:4-linkages occur separately in distinct groups in the barley β-glucosan.

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Protein of Pasture Plants

CYTOPLASMIC PROTEIN OF WHITE CLOVER AND ITALIAN RYEGRASS

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Studies of the proteins of the leaves of pasture plants have in general been confined to estimation of the amount of crude protein present, and analysis of this crude protein in terms of amino acid content. (For reviews see Chibnall, 1939; Lugg, 1949.) For this type of work, quantitative separation of the total protein of the leaf is desired, and the physical state of the extracted protein is relatively unimportant. The methods used frequently lead to denaturation, and very little has been learned of the properties of the native protein of the leaf cells.

The methods of extraction which are used when the protein is to be recovered without denaturation have recently been reviewed by Wildman & Jagendorf (1952), who discuss at length the work of the Californian school on the physical chemistry of the leaf proteins of several higher plants. They draw attention to the compromise aimed at in devising methods severe enough to cause rupture of a reasonably large percentage of leaf cells, yet not so severe as to denature the soluble protein within these cells. The essence of the methods used is cell rupture by mechanical stress, the acid of the liberated vacuolar fluid being neutralized by extracting into buffer solution. The protein is thereby subjected to no chemical denaturant in extraction, and is protected from acid precipitation when liberated; but no safeguard can yet be offered against changes in protein structure brought about by the disorganization of cell contents, soluble and in-

soluble, which results from the severity of the method.

These limitations have been accepted in this work, in which a study is made of the soluble proteins extracted from pasture plants, mainly white clover (*Trifolium repens*) and to a less extent Italian ryegrass (*Lolium multiflorum*).

MATERIALS AND METHODS

Clover. White clover was grown in plots out of doors under normal pasture conditions. Plants more than 6 months old were not used. In cutting the clover, as little stalk as possible was included with the leaves.

Ryegrass. Italian ryegrass was grown in boxes in open frames, and kept watered with adequate nutrient solution. Grass was taken for several regrowths, but new boxes were regularly planted and old plants were not used.

Electrophoresis. Extracts and fractions were analysed by electrophoresis at 0·5° in the Tiselius-type apparatus manufactured by LKB-Produkter Ltd. Unless specified, all runs were carried out in phosphate buffer (Na_2HPO_4, KH_2PO_4), pH 7·5, ionic strength (I) 0·1. (Mobilities are expressed in units of 10^{-5} cm.^{2}v^{-1} sec.$^{-1}$.) When particularly densely coloured solutions were run, it was found convenient to use a tungsten incandescent lamp in conjunction with an infrared filter (Ilford no. 207) and Kodak 35 mm. infrared film. So that the pattern could be observed by visible light, the infrared filter was placed immediately in front of the camera shutter, behind the reflex mirror. No refocusing of the optical system was necessary.

Dialysis of extracts before electrophoresis was carried out at 2° in Cellophan tubing on a rocking dialyser against repeated changes of buffer. In all cases the establishment of equilibrium was confirmed by conductivity measurement.

Paper electrophoresis. This was carried out in an apparatus somewhat similar to that of Vesselinovitch & Funnell (1954), in which the paper lies in a horizontal plane between two flat plates, supported by a series of insulated points.

Diffusion constants. These were measured in the LKB electrophoresis apparatus, the change in shape of the schlieren curve being followed for 3 days at 5°.

Ultracentrifugal analyses. Centrifuging for analytical and preparative purposes, at 50000–200000 *g*, was carried out in an air-driven ultracentrifuge constructed for this Division by Dominion Physical Laboratory.

Absorption spectra. Visible and ultraviolet absorption spectra were measured in a Beckman DU quartz spectrophotometer.

Determination of N *and* P. N was determined by micro-Kjeldahl estimation, with $CuSO_4$ as a catalyst in the digestion mixture. P was determined by the method of Allen (1940).

Phosphatase activity. This was estimated by measuring the liberation of inorganic phosphate in 30 min. from 1% (w/v) β-glycerophosphate at pH 6 in 0·15M-KOH–maleic acid buffer at 30°.

Ribonucleic acid (RNA). The RNA content of samples was estimated by precipitation with trichloroacetic acid, washing, and extraction for 18 hr. in the cold in 10% (w/v) $HClO_4$, according to the method of Ogur & Rosen (1950). The absorption spectrum of the extract was measured in the range 300–240 mμ., to confirm the presence of the typical curve for RNA with maximum at 260 mμ. The concentration of RNA was then expressed directly as the optical density at the maximum (D_{260}) of the extract derived from 1 ml. of sample, after correction for dilution during extraction and measurement.

Preparation of extracts

In the method finally adopted, the leaves were ground into buffer solution by mechanical pestle and mortar action in an end-runner mill (Pascall Engineering Co., Ltd.) operated in a cold room at 2–3°. This method, which has been used by Crook (1946), was relatively rapid, and did not lead to local heating or severe frothing, as can occur with blending or extraction with a colloid mill. The convenient capacity of the end-runner mill was 100–300 ml. of buffer, and it was found most efficient to grind fresh leaves of about half the weight of buffer in a single stage. After 5–10 min. the leaves were reduced to a slurry, which was filtered in the cold through a basket centrifuge lined with Whatman 3MM filter paper. This extract, free from cell-wall debris and gross solid matter, could be returned to the mill for a second load of leaves. By this means, even without the addition of abrasive materials such as sand, 80–90% of the total nitrogen of the leaf was regularly liberated into the extract; when two stages of extraction were used, a concentration of soluble protein suitable for electrophoretic and ultracentrifugal examination was readily obtained.

In most cases phosphate buffer (Na_2HPO_4–KH_2PO_4, pH 7·5; I 0·2) was used for extraction, though 0·15M-KOH–maleic acid buffer (pH 7·4) and borate buffer (H_3BO_3–NaOH–NaCl, pH 8·0; I 0·1) were occasionally used. Provided that the pH remained above 6·5 during extraction, no significant difference in yield or in any measured property of the extract was observed.

After the leaves had been ground and the slurry filtered, the resultant extract from both clover and ryegrass was an opaque green liquid, heavily contaminated with insoluble matter that was largely derived from the fragmentation of chloroplasts during extraction. The bulk of this could be removed by centrifuging at 25000 *g* at 0° for 40 min. (International Model PR2, high-speed head), and this centrifuging was regularly carried out immediately after extraction. For the remainder of this paper, the terms 'clover extract' and 'ryegrass extract' refer to the supernatant produced by this centrifuging. The nitrogen thus removed as insoluble material was between 15 and 25% of the total of the crude extract. The range in amount of this loss was due to different degrees of prior removal of solids by the basket centrifuge, as the cake of cell-wall debris acted as a filter of variable efficiency. If the slurry of ground leaves was pressed through cheesecloth instead of passing through the basket centrifuge, the nitrogen removed at 25000 *g* rose to 28–34%.

RESULTS

Clover extract. This was a green–brown transparent solution which exhibited strong scatter of light caused by highly dispersed insoluble matter. This could be removed by centrifuging at 50000 *g* for 15 min. with loss of a further 3–4% nitrogen, yielding a clear brown liquid, but in most cases this step was not taken. Attempts were made to reduce contamination with insoluble green material by

grinding into buffer containing 0·5 M sucrose to reduce osmotic damage to the chloroplasts (Neish, 1939). The chlorophyll content of the extract fell by 25–30 %, but the efficiency of extraction was also reduced, so that the ratio of chlorophyll to nitrogen fell by only 5–8 %. The mechanical stresses produced during grinding are probably sufficient to disrupt a large proportion of the chloroplasts, despite protection from osmotic damage. In view of the small improvement produced, and the difficulties introduced by the high concentration of sucrose, plain buffers were regularly used for extraction.

The brown colour of the extract appeared to be due to atmospheric oxidation of soluble constituents liberated from the cell, and could be reduced by working rapidly in the cold. On standing in air, even at 2°, the solutions darkened perceptibly at the surface over a period of hours. This oxidation could be almost completely prevented by extracting into buffer containing either sodium dithionite (0·05 M) or ascorbic acid (0·05 M). The extracts in these cases were of a pale-green colour, and browning at the air surface was markedly inhibited. In the experiments described in the remainder of this paper, however, these agents were not included in the extraction medium.

When reducing agents were not used, clover extract after removal of solids at 50 000 ***g*** produced an absorption spectrum as shown in Fig. 1. The slight maximum at 280 mμ., and the rapid rise at 240 mμ., are typical of proteins containing aromatic amino acids, while the general rise in the absorption with decreasing wavelength in the region 400–300 mμ. appeared to be associated with the brown pigment produced by oxidation. Unfortunately both sodium dithionite and ascorbic acid absorb strongly in the ultraviolet region, so that spectra of extracts in these media could not usefully be compared with those of normal extracts.

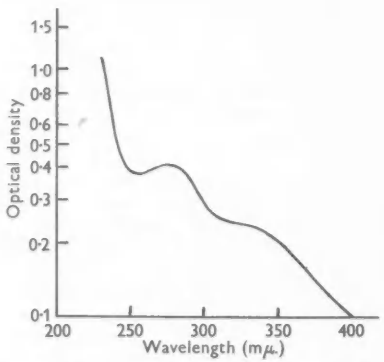

Fig. 1. Ultraviolet absorption spectrum of clover extract.

It was found that the height of the maximum at 280 mμ. was a useful measure of removal of pigmented material during fractionation of the extract. This height was expressed as the ratio of the optical densities at 280 and 310 mμ. (D_{280}/D_{310}). The value of this factor decreased from about 1·6 for fresh clover extract to 1·4 as oxidative darkening proceeded.

The protein contained in clover extract was apparently relatively stable, and at pH values between 6·5 and 7·5 could be stored at $-20°$ for a few weeks or at $+2°$ for a few days without becoming insoluble. Storage of the extract at $-20°$ led to aggregation of the remaining insoluble matter, which could then readily be removed at 5000 ***g***.

From 80 to 90 % of the nitrogen of clover extract was precipitable with 10 % (w/v) trichloroacetic acid. This percentage dropped to 70–75 % after 4 days at 2°, owing presumably to the breakdown of high-molecular-weight material. It was therefore necessary to work quickly when fractionating the extract, and fractionation was normally complete within 2–4 hr. of extraction.

Clover extract contained RNA in solution. Its concentration was estimated after precipitation in terms of the ratio D_{260}/N, this being the ratio of the optical density at 260 mμ. of the extracted RNA to the nitrogen content in mg./ml. of the solution from which this was taken. The value of the ratio for fresh clover extract lay between 4 and 6. If the extract was kept at 2°, the amount of RNA which could be estimated by extraction from a trichloroacetic acid precipitate progressively decreased. This was presumably due to hydrolytic breakdown into soluble units, as in 0·15 M potassium maleate buffer (pH 7·5) the loss of RNA was accompanied by release of inorganic phosphate. With different samples of extract the apparent rate of hydrolysis was not regular, between 40 and 70 % of the RNA disappearing in 4 days. With uncentrifuged clover extract, the amount of RNA extractable from the trichloroacetic acid precipitate, which included insoluble matter as well as components from solution, fell more rapidly than with centrifuged extract, and the rate of fall was further increased if the integrity of cell components was partially preserved by extracting into buffer which contained sucrose.

The value of the ratio D_{260}/N gave an estimate of the percentage of nitrogen present as RNA. According to Ogur & Rosen (1950) the extinction at 260 mμ. per mole of P of RNA treated with perchloric acid is 10 800. This corresponds to a value for D_{260}/P of 348; if the N:P ratio of 15·3:8·0 for purified yeast RNA is used (Vischer & Chargaff, 1948), D_{260}/N for the pure acid is 182. The values found here show that in clover extract 2–3·5 % of the nitrogen is present in the form of RNA.

After dialysis, clover extract in the electrophoresis apparatus revealed a single component which was rather asymmetric, particularly in the descending boundary (Fig. 2*a*, *b*), with a mobility of 5·5–6·0 units in phosphate buffer at pH 7·5 (*I* 0·1). This mobility decreased with pH, falling to 4·2 units at pH 6·0, below which the protein tended to precipitate during the run. The bulk of the brown pigment migrated with the main boundary, though a small fraction regularly ran ahead of the ascending boundary with mobility of 12–13 units. The chloroplast fragments remaining in the extract also migrated with the main boundary, slightly behind its maximum ordinate.

The abnormally large boundary anomalies found for plant cytoplasmic proteins in phosphate buffers by Wildman, Cheo & Bonner (1949) were observed, and associated with these was an abnormally high ratio of ascending to descending mobility. These abnormalities decreased if the extract was dialysed for several days before electrophoresis, and were eliminated if the sample examined was reconstituted after freeze-drying.

In the ultracentrifuge, clover extract separated into three components (Fig. 2*c*, *d*): a small fraction (3–5 %) with sedimentation constant (S_{20}) 65–70 Svedberg units (referred to subsequently as *H*), a monodisperse fraction (35–40 %) with S_{20} approx. 17 units (fraction I), and a polydisperse residuum of material of lower molecular weight (52–58 %) with mean S_{20} approx. 4 units (fraction II). The proportions of these components found by analysis of ten extracts from mature clover were within the ranges quoted. It was evident that the brown pigment was associated primarily with fraction II, no change in colour occurring at the boundaries due to fraction I or the *H* component.

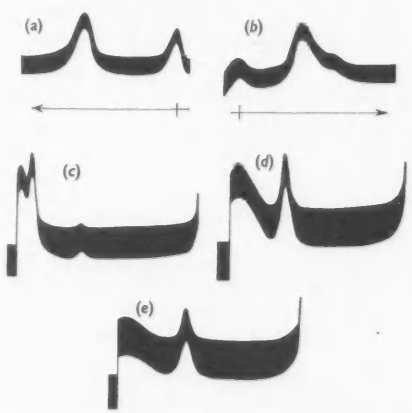

Fig. 2. Electrophoresis and ultracentrifuge diagrams of extracts. (*a*) and (*b*), electrophoresis diagrams of clover extract, ascending and descending boundaries respectively. 5·5 v/cm. for 210 min. (*c*) and (*d*), ultracentrifuge diagrams of clover extract, at 45 000 rev./min. during acceleration, and after 10 min. at 60 000 rev./min. respectively. (*e*), ultracentrifuge diagram of ryegrass extract, after 10 min. at 60 000 rev./min.

The ultracentrifuge thus proved to be more useful than the electrophoresis apparatus in following the fractionation of extracts. It also revealed that the apparent stability of the extracts at 2° was deceptive. Although the *H* fraction was stable for several days, it was rapidly lost on dialysis. At pH values between 6·5 and 7·5 the concentration of fraction I slowly decreased (20 % loss in 4 days), presumably because of enzymic decomposition, as no aggregation was evident. At pH 6·0, however, aggregation occurred, leading to 50 % loss of fraction I in 3 days. Similarly storage at −20° led to considerable loss of fraction I and *H* component due to aggregation, particularly if the extract was frozen before centrifuging at 25 000 *g*.

Extraction of clover leaves into buffer containing sodium dithionite to inhibit oxidation resulted in complete loss of *H* and 16 % loss in fraction I. The effect of using buffer containing ascorbic acid was not determined.

Ryegrass extract. The properties of ryegrass extract after centrifuging at 25 000 *g* were broadly similar to those of clover extract. The brown pigmentation was much more prominent, however, obscuring the green of the contaminating chloroplast fragments. By extracting into buffer containing 1 % of sodium dithionite the browning could be almost completely prevented, but this agent was not used in experiments described subsequently.

Ryegrass extracts were less stable than those from clover. Obvious aggregation took place in neutral buffer after a few days at 2°, and after a week at −20° the bulk of the protein could be sedimented at 25 000 *g*. The extracts contained RNA in solution, with values of D_{260}/N between 4 and 7. This RNA broke down at much the same rate as in clover extract.

Electrophoresis revealed a pattern similar to that found with clover, i.e. an asymmetric single peak with a mobility of 6·5–7 units. The bulk of the pigment migrated with the main peak, a small amount running ahead of the ascending boundary with a mobility of 12–13 units. The ultracentrifuge pattern (Fig. 2*e*) revealed components corresponding to fraction I (35–45 %) with S_{20} approximately 16 Svedberg units and to fraction II (55–65 %) with mean S_{20} between 3 and 4 units. A component corresponding to *H* was observed only occasionally, and then only as a trace. The aggregation of protein of the extract on standing at 2°, or on storing at −20°, led to rapid decrease in fraction I.

Table 1. *Ultracentrifugal fractionation of clover extract*

For definition of D_{260} and D_{260}/N see text.

(*a*) *S*1 produced by clarification of clover extract at 100000 *g*; fractionated at 200000 *g*

Fraction	Vol. (ml.)	N (mg./ml.)	D_{260}/N	Fraction II*	% Fraction I†	Component *H*
*S*1	49	2·01	3·8	2·57	41	+
*S*2*a*	28	1·01	0·7	1·72	7	–
*S*2*b*	20	2·57	1·7	2·90	39	–
*S*2*c*	5	2·30	2·3	0·84	82	–
*P*2	5	1·31	18·3	0·50	81	+ +

(*b*) *S*1 produced by clarification of clover extract at 50000 *g*; *S*1 fractionated at 100000 *g*, and *S*2 at 200000 *g*

Fraction	Vol. (ml.)	N (mg./ml.)	D_{260}/N	Fraction I*	Component *H**
*S*1	73	2·64	5·3	—	—
*S*2	72	2·44	3·1	—	—
*P*2	2·8	3·58	46	0·96	2·01
*S*2	65	2·44	3·1	—	—
*S*3*a*	50	1·53	0·9	—	—
*S*3*b*	13·5	3·44	1·5	—	—
*S*3*c*	4	3·50	1·6	4·84	0
*P*3	3	4·34	19·5	6·15	1·05

* Expressed in arbitrary units derived from areas on ultracentrifuge diagrams.
† Expressed as percentage of total area.

Ultracentrifugal fractionation

Clover extract. An attempt was made to separate fractions I, II, and the *H* component by centrifuging at high speeds. An extract of clover in phosphate buffer (resultant pH 6·8, *I* 0·2) was spun at 100000 *g* for 15 min. The resulting supernatant (*S*1) was spun at 200000 *g* for 50 min., after which the centrifuge tubes contained a transparent sediment of gel-like material as well as a small well-defined layer of dense solution at the bottom. The tube contents were separated into four fractions: the upper three-fifths (*S*2*a*), the lower two-fifths (*S*2*b*), the dense layer (*S*2*c*) and the sediment (*P*2). The dense layer was diluted with buffer and the sediment dissolved by shaking for 30 min. into buffer, giving a solution from which a small precipitate could be removed at 10000 *g*. The results of analyses of these fractions are set out in Table 1 (*a*). A similar fractionation carried out on clover extract in 0·5 M potassium maleate buffer (pH 7·5) gave almost identical results.

The expected partial separation of fractions I and II was achieved, but recovery of *H* was very poor. Only in *P*2 was it observed, and here in a partially degraded form. The recovery of RNA was only 67 %. It was evident that some RNA, and probably some *H* component, were lost during clarification at 100000 *g*. This loss was prevented by clarifying clover extract at 50000 *g* for 15 min. and withdrawing the upper four-fifths of the supernatant (*S*1), thus avoiding contamination with larger particles. This *S*1 was spun at 100000 *g* for 40 min., giving a sediment of gel-like material (*P*2), after which the supernatant (*S*2) was spun at 200000 *g* for 40 min., yielding fractions *S*3*a*, *b*, *c* and *P*3 corresponding closely to those of the first method. Both sediments redissolved in buffer when the suspensions were shaken for 30 min. The results of the analysis of these fractions are given in Table 1 (*b*).

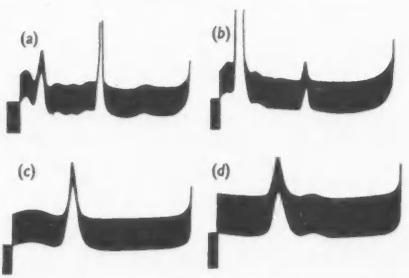

Fig. 3. Ultracentrifuge diagrams of fractions prepared by centrifuging (see Table 1). (*a*), clover *P*2; 42000 rev./min. 10 min. (*b*), clover *P*3; 42000 rev./min. 10 min. (*c*), clover *S*3*c*; 60000 rev./min. 10 min. (*d*), ryegrass *S*2*c*; 60000 rev./min. 10 min.

In this case 97 % recovery of RNA was obtained in the fractionation of *S*1, and 87 % in fractionation of *S*2. In the ultracentrifuge, *P*2 showed a high concentration of *H*, as well as some material sedimenting between this and fraction I. *P*3 showed a much smaller proportion of *H*. Fig. 3 shows the ultracentrifuge patterns produced by *S*3, *P*2 and *P*3.

The ultraviolet absorption spectrum of *P*2 and *P*3 showed a broad maximum at 260 mμ., but the

spectrum of $S3c$ was similar to that of unfractionated extract, with a somewhat enhanced maximum at 280 mμ. ($D_{280}/D_{310} = 2{\cdot}2$).

Samples of $S3c$, $P2$ and $P3$ were analysed by paper electrophoresis, with Whatman 3MM filter paper, and sodium phosphate–potassium phosphate buffer (pH 7·5, I 0·05). A field of approximately 2v/cm. was applied for 16 hr., after which the paper was cut laterally into strips and eluted, and the absorption spectra of the eluates were analysed for RNA and for protein. Although RNA has a much higher specific absorption than protein, the ratio of the optical densities at 260 and 280 mμ. gave a qualitative estimate of the proportion of RNA to protein. Protein was clearly demonstrated in $S3c$ and $P3$, but only a trace was found in $P2$; RNA was found only in $P2$ and $P3$.

Some tailing of protein occurred, but the bulk had migrated 10 cm. under the conditions of the experiment. The RNA migrated slightly ahead of this, overlapping at about 10 cm.

Ryegrass extract. This was fractionated at 200 000 g by the first method used with clover. The results were similar, the expected concentration of fraction I occurring in the dense layer at the bottom of the tube ($S2c$), as illustrated in Fig. 3 (*d*). For the sediment ($P2$) the value of D_{260}/N was 18; for all other fractions it was below 2.

Salt fractionation

Clover and ryegrass extracts were fractionated at pH 6·8 by precipitation with sodium sulphate. As the concentration of added salt increased, the precipitated nitrogen increased without any abrupt discontinuity, but it was found that precipitation with 12, 18 and 25 % Na_2SO_4 (w/v) gave convenient separation into sufficiently well-marked fractions. The precipitates were named $P1$, $P2$ and $P3$ respectively, and the final supernatant $S3$. Each precipitate was shaken with enough phosphate buffer (pH 7·5, I 0·1) to give a volume one-tenth of that of the original extract, and centrifuged at 5000 g to remove insoluble material. The precipitates dissolved readily, with the exception of $P1$, from which a green sediment of aggregated particles was obtained. The results of typical experiments with clover and ryegrass are given in Table 2.

Poor recovery (18 %) of RNA was obtained, and no trace of H was found in any fraction. It was evident from electrophoretic analysis of the precipitates that no separation of fractions I and II took place during migration, and the pattern gave no clear indication of their relative proportions. A high proportion of fraction II was evident only from an increased spreading of the peak. This spreading could be reversed by reversing the direction of the current in the cell, so it was due to inhomogeneity rather than to true diffusion.

After concentration, $S3$ (clover) showed on electrophoresis a diffuse peak with a mobility of approximately 7 units, with a large amount of polydisperse material behind a small peak with a mobility of 12 units.

An indication of the change which takes place in clover extract on standing was given by the different distribution which resulted from salt precipitation carried out after 3 days at 2°. $P1$ then contained 11 % of the nitrogen in the form of heterogeneous material which sedimented at 100 000 g in the ultracentrifuge, and a high proportion of fraction I occurred in $P3$ rather than $P2$. Despite the obvious change of character of $P1$, it migrated as a single symmetrical peak on electrophoresis.

Acid fractionation

Extracts of clover and ryegrass were fractionated by addition of successive amounts of 0·2 N-HCl at 2°. Each precipitate was shaken with a volume of phosphate buffer (pH 7·5, I 0·2) equal to one-tenth of that of the original extract, and the mixture was spun at 5000 g to remove insoluble material. The results are set out in Table 3.

As with salt precipitation, no well-marked discontinuity of precipitation was noted as the pH was lowered; the fractions selected in Table 3 are typical of the separation obtained by this method.

The recoveries of RNA were 72 % with ryegrass and 24 % with clover. Only traces of H were found in the clover fractions; none was observed in ryegrass fractions.

Table 2. *Salt fractionation of clover and ryegrass extracts*

For conditions see text.

	Clover					Ryegrass			
Fraction	Concn. of Na_2SO_4 (%, w/v)	N as % of total	D_{260}/N	D_{260}/D_{310}	Fraction I (%)*	Concn. of Na_2SO_4 (%, w/v)	N as % of total	D_{260}/N	Fraction I (%)*
Extract	0	100	4·8	1·5	38	0	100	4·4	45
$P1$	12	4·5	0·6	1·3	27	12	24	0·7	42
$P2$	18	34	0	2·2	71	18	12·5	0	57
$P3$	25	8·5	0	1·4	21	25	24	0	67
$S3$	—	24	2·4	1·2	—	—	35	1·9	—

* Expressed as a percentage of the total area on ultracentrifuge diagrams.

Table 3. *Acid fractionation of clover and ryegrass extracts*

For conditions see text.

Clover					Ryegrass				
Fraction	pH	N as % of total	D_{260}/N	Fraction I (%)*	Fraction	pH	N as % of total	D_{260}/N	Fraction I (%)*
Extract	7·0	100	5·8	38	Extract	7·0	100	6·5	45
*P*1	5·25	37	2·3	58	*P*1	5·2	1·5	3·0	—
*P*2	4·6	14	3·6	24	*P*2	4·4	15	4·1	40
*S*2	—	31	0	—	*P*3	4·0	35	10·6	47
					*S*3	—	40	2·5	—

* Expressed as a percentage of the total area on ultracentrifuge diagrams.

Table 4. *Ethanol fractionation of clover and ryegrass extracts*

For conditions see text.

Fraction	Ethanol (% by vol.)	N as % of total	P as % of total	D_{260}/N	Fraction I (%)*
		Clover			
Extract	0	100	100	4·0	39
*P*1	11·3	12·5	11·0	3·6	33
*P*2	13·9	9·0	7·0	5·1	32
*P*3	16·5	14·0	10·0	6·2	41
*P*4	19·1	15·0	10·0	6·7	50
*P*5	21·6	11·5	7·0	6·3	63
*P*6	26	8·0	4·5	5·4	59
*S*6	—	22·5	45·0	0	—
		Ryegrass			
Extract	0	100	100	5·5	35
*P*1	11·0	3·5	2·0	4·5	—
*P*2	16·0	13·5	5·5	5·4	47
*P*3	20·5	18·0	6·5	12·1	50
*P*4	24·5	10·0	6·0	19·4	52
*P*5	32	10·0	12·0	12·0	35
*S*5	—	39·0	61·5	1·5	—

* Expressed as a percentage of the total area on ultracentrifuge diagrams.

Ethanol fractionation

Fractionation of protein solutions by precipitation with organic solvents at low temperatures has been recognized in recent years as a powerful and flexible technique. So far, it does not seem to have been used with proteins extracted from plant leaves. Experiments were accordingly carried out, with ethanol as a precipitant, to find conditions under which satisfactory fractionation of clover and ryegrass extracts could be achieved.

As expected, it was necessary to keep the temperature below 0° if undenatured precipitate was to be obtained. At 2–3°, most of the precipitate formed would not redissolve, but if the temperature was lowered to −5° as the ethanol was added, the precipitate seemed undenatured, and redissolved readily in buffer. It was also necessary to work at a pH below 6·3 to give precipitation at reasonable concentrations of ethanol. At pH 6·8–7·0 no precipitate formed at ethanol concentration as high as 70 % by volume. If the pH of a solution containing this concentration of ethanol was lowered, protein was precipitated in a denatured form even at −8°, and chlorophyll which was present in the extract in dispersed chloroplast fragments remained in solution.

Proteins from clover and ryegrass extract in 0·15 M potassium maleate buffer were precipitated at pH 6·1 by addition of successive amounts of 95 % (v/v) ethanol, the temperature of the extract being lowered to −5° during the first addition, and being maintained there for subsequent additions. The precipitates were sedimented at 4000 *g* with the centrifuge rotor chamber at −10°, and redissolved in one-tenth of the original volume of 0·15 M potassium maleate buffer, pH 7·5, at 2°. The results are set out in Table 4.

Complete recovery of RNA was obtained, with good recovery of N and P. Peaks corresponding to *H* in the ultracentrifuge diagrams of precipitates 2–5 (clover) were observed, but these were too small to measure accurately.

Purification of fraction I

Two samples of fraction I from clover extract, each of which contained less than 3 % of fraction II, were prepared by a single stage of precipitation, followed by two stages of fractional centrifuging.

In the first method, an ethanol precipitate equivalent to $P4+P5+P6$ of Table 4 was prepared, redissolved in phosphate NaCl buffer (Na_2HPO_4–KH_2PO_4, I 0·1; NaCl, I 0·2; pH 7·5), and centrifuged at 200000 g for 45 min., giving a sediment and a layer of concentrated solution at the base of the tube similar to that found in the ultracentrifugal fractionation of clover extract. This concentrated solution was withdrawn, diluted with phosphate–NaCl buffer, and centrifuged a second time at 200 000 g for 45 min., giving a trace of sediment and a layer of concentrated solution. This solution, diluted with buffer, gave the ultracentrifuge pattern shown in Fig. 4*a*.

The second method was similar, except that a salt precipitate equivalent to $P2$ of Table 2 was used as the starting material. The resulting fraction I appeared identical with that of the first method. Neither protein contained measurable amounts of RNA. The salt-precipitated preparation showed the value of 3·65 for D_{280}/D_{310} from the absorption spectrum, and the electrophoretic mobility was 5·8 units. A slight amount of reversible spreading took place during migration, showing that the preparation was not homogeneous. The stability of this purified fraction I was considerably greater than in clover extract, and it could be stored at −20° for 10 days without change.

The sedimentation constants (S_{20}) in Svedberg units at 0·5 % (w/v) protein concentration for the two preparations were 16·4 (ethanol precipitate) and 16·7 (salt precipitate). In calculating these results, the value of 0·75 was assumed for the partial specific volume of the protein, and no extrapolation to infinite dilution was made. A single measurement of S_{20} at 2 % (w/v) protein concentration ($S_{20}=16·2$) differed only slightly from that at 0·5 %, so that the error in omitting this extrapolation is small. The diffusion constant for each preparation was determined at 0·5 % (w/v) concentration, with the following results for D (cm² sec.$^{-1}$): $2·60\times10^{-7}$, for the ethanol precipitate; $2·75\times10^{-7}$, for the salt precipitate.

Ultracentrifugal analyses were made before and after diffusion to confirm absence of aggregation or breakdown into smaller units. From these results, the value 0·75 again being used for partial specific volume, the molecular weights were calculated to be $6·2\times10^5$ for the ethanol precipitate and $5·9\times10^5$ for the salt precipitate.

In view of the lack of precision in determining the diffusion constant by the schlieren-curve method without a specially designed cell, the difference between these values is probably not significant.

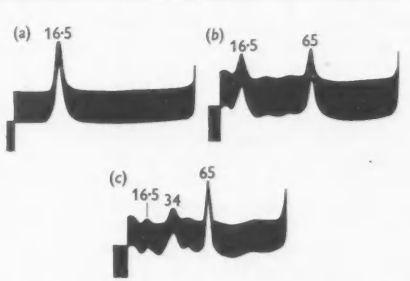

Fig. 4. Ultracentrifuge diagrams of fractions prepared from clover extract. (The approximate sedimentation constants are indicated in each diagram.) (*a*), Fraction I; 60000 rev./min. 10 min. (*b*), component, once sedimented; 42000 rev./min. 10 min. (*c*), H component, twice sedimented; 42000 rev./min. 10 min.

Concentration of the H component

Fractions containing a high proportion of component H were prepared by adding ethanol to a concentration of 21 % (by vol.) to clover extract at pH 6·1, producing a precipitate equivalent to $P1$–$P5$ of Table 4. After the precipitate had been redissolved in buffer at pH 7·5, the solution was clarified at 50000 g for 15 min., and H sedimented by centrifuging at 100000 g for 50 min. The sediment contained 52 % of H (Fig. 4 *b*) and a high proportion of RNA ($D_{260}/N=40$). Analysis of this preparation by paper electrophoresis under the conditions described above for $P2$ of Table 1 (*b*) revealed a large amount of RNA migrating from 10 to 16 cm. from the origin, and a trace of protein at 10 cm.

In establishing a suitable ionic content of the solution to carry out electrophoresis of this preparation in the Tiselius apparatus, it was necessary to avoid dialysis, which causes rapid breakdown of H. Accordingly, the once-sedimented preparation was diluted with a relatively large volume of the buffer to be used ($Na_2HPO_4+KH_2PO_4$, pH 7·5, I 0·1), and recentrifuged at 100 000 g for 50 min. This twice-sedimented preparation contained, besides a high content of H and RNA, a new major component with sedimentation constant of 34 Svedberg units (Fig. 4*c*). Electrophoresis produced two distinct boundaries with mobilities of 5·5 and 8·3 units.

Qualitative analysis of this fraction for pentoses by the method of Ceriotti (1955) indicated the presence of ribose, but no detectable deoxyribose.

Phosphatase activity

Fractions of clover and ryegrass extracts in 0·15 M potassium maleate buffer, prepared by centrifuging as in Table 1 (*a*) and by ethanol precipitation as in Table 4, were examined for phosphatase activity. No great concentration of activity was found in any one fraction; high activity was associated with high content of fraction II, but the relationship was not linear. Complete recovery of activity was found in the centrifuged fractions; for ethanol precipitation the supernatant with a high content of ethanol was not assayed, but the precipitates contained 77 % (for clover) and 74 % (for ryegrass) of the activity.

DISCUSSION

One of the most striking points which emerges from this work is the similarity in many respects with the results obtained by Wildman and co-workers (Singer, Eggman, Campbell & Wildman, 1952; Eggman, Singer & Wildman, 1953) in their studies on a range of higher plants, chiefly tobacco and spinach. The physicochemical properties of proteins obtained from the leaf cells of these plants were remarkably similar, and the similarity extends to the pasture plants considered in this paper.

On the other hand, it has been suggested (Wildman & Jagendorf, 1952), on the basis of the properties of extracts from corn and oat leaves, that monocotyledons might differ from dicotyledons in that no fraction I protein was found in their leaves. The results given here for Italian ryegrass, as well as ultracentrifugal analysis of extracts from short-rotation ryegrass, perennial ryegrass, cocksfoot and wheat seedlings, show that in these monocotyledons a protein corresponding to fraction I is present to as great an extent as in dicotyledonous leaves. Thus although some genera of monocotyledons may not contain fraction I, there is no fundamental difference between the two classes in this respect.

The sedimentation constant of fraction I from clover falls within the range given for plants studied by Singer *et al.* (1952). Although these workers did not obtain this protein in a form sufficiently pure to enable the diffusion constant to be measured, their estimate of 600000 for the molecular weight agrees well with that found here.

Components corresponding to *H* have not been described in extracts from other plants, though material with the considerably lower sedimentation constant of 25 units has been described (Singer *et al.* 1952). Components of high sedimentation constant have been produced as artifacts by polymerization of fraction I as a result of repeated sedimentation (Eggman *et al.* 1953), but the *H* component, present in uncentrifuged clover extract, does not arise in this way. Artifacts of this type have been observed in this work (e.g. Figs. 3*a*; 4*b*, *c*), but the amount of *H* in these fractions may be accounted for by concentration of material already present.

When prepared by the methods used here, a fraction rich in *H* has always contained a high concentration of RNA, and it was thought that *H* might be a nucleoprotein of high molecular weight, possibly corresponding to the fraction NP of tobacco leaves described by Pirie (1950). Electrophoresis of concentrates showed two boundaries, as with Pirie's fraction, but attempts to isolate and characterize the components producing the two boundaries have not been successful. Thus the nature of *H* has not been established. It has not been prepared in a pure form, and has been concentrated only by methods involving fractional centrifuging, so that the association with RNA may be due only to similar sedimentation rates. Although both *H* and RNA are unstable, RNA persisted in solution under conditions which led to loss of *H*. This observation, in conjunction with the fact that ryegrass extracts have a high RNA content, yet often no *H* component, suggests that *H* and RNA may not be related.

The distribution of RNA found in clover and ryegrass extracts agrees with that found by Pirie (1950) for tobacco in that the bulk is sedimentable with centrifugal fields of the order of 100 000 *g*. With the possible exception of the *H* component, no homogeneous fraction identifiable with his nucleoprotein NP has been found by ultracentrifugal or electrophoretic analysis. The RNA appears to be extracted as polydisperse material of high molecular weight.

It has been suggested by Wildman & Jagendorf (1952) that Pirie's NP results from aggregation of fraction I in the acid conditions brought about by extraction without the use of buffers. Aggregation of this type has been successfully avoided in this work, yet the bulk of the RNA readily sediments in centrifugal fields which will sediment fraction I only very slowly.

Complete removal of RNA at 100 000 *g* was not expected; it is known that leaf extracts contain a ribonuclease (Parker, 1952) by which initially large molecules of RNA are progressively broken down to fragments, and during extraction of cell contents this will take place to some extent. Only by working rapidly in the cold has it been possible to sediment a high proportion of RNA; delay of even a few hours greatly reduces the yield.

The abnormally large boundary anomalies and high ratio of ascending to descending mobility observed in electrophoretic analysis of clover extracts in phosphate buffer are possibly due to the presence of RNA in these extracts. The bound phosphate groups of RNA, dissociated at pH 7·5,

would be expected to reduce the conductivity established by Donnan equilibrium on dialysis, thus producing the abnormalities observed. Support for this is found in the fact that the abnormalities disappear when RNA is absent. The difference between the conductivity of the extract and that of the dialysate at equilibrium was also found to depend on the presence of RNA, the difference decreasing as RNA disappeared. On the basis of this suggestion, the finding of Wildman *et al.* (1949) that electrophoresis of extracts in buffer such as cacodylate showed no abnormalities might be explained as due to accelerated enzymic removal of bound phosphate groups in the absence of inhibition by excess of phosphate ions in the buffer.

Eggman *et al.* (1953) described fraction I, prepared from spinach and tobacco extracts by fractional centrifuging, as a nucleoprotein, and believe that all the soluble RNA of the extracts is associated with this protein. The results obtained here indicate that fraction I from clover is not associated with significant amounts of RNA. The evidence for this may be outlined as follows.

In fractional centrifuging, the RNA content of the fractions bears no relationship to the content of fraction I. This is most clearly seen in fractions *P*2, *P*3 and *S*3*c* of Table 1 (*b*), where the quotients RNA/fraction I (expressed as D_{260}/fraction I area) are 172, 14 and 1·2 respectively. Residual RNA in other fractions is probably due to contamination with lower-molecular-weight products of ribonuclease digestion.

The sedimentation constants of fraction I preparations did not vary with the RNA content of the samples. Thus the mean value for the two most highly purified samples, containing no RNA, was 16·55; that of three samples prepared by single-stage centrifuging [equivalent to *S*2*c* of Table 1 (*a*)] was 16·5; and the value determined for the fraction I component of *P*2 (Fig. 3*a*) with a very high RNA content was 16·2. This constancy of value indicates lack of association between fraction I and RNA molecules.

In preparing samples of fraction I for analysis, Eggman *et al.* (1953) separated a fraction by centrifuging which was apparently equivalent to *P*2 of Table 1 (*a*). This sediment, containing both RNA and fraction I, produced aggregated polymers of fraction I on recentrifuging. Such polymers have not been produced in this work on recentrifuging of fraction I preparations, provided contamination with large amounts of RNA was avoided. Recentrifuging of a preparation rich in RNA produced aggregation of this type (Fig. 4*c*); in this case the polymer was considerably in excess of the unaggregated form. Indication of formation of smaller amounts of similar aggregates is seen in Fig. 3*a*, *b*. Paper electrophoresis of fractions rich in RNA indicated that the protein component (fraction I) did not migrate in close association with RNA, although the leading edge of the protein band overlapped the rear of the RNA. From all these considerations it is concluded that fraction I of clover is not a nucleoprotein.

Of the various methods of fractionation used in this work, centrifuging is that least liable to modify the various components of cytoplasmic extracts. It does not, however, lend itself readily to fractionation of large quantities with high yields. Salt precipitation is a convenient method of producing quantities of partially purified fraction I, but RNA and the *H* component are not recovered. Multiple-stage salt precipitation leads to decreasing recovery, and even in a single stage Wildman & Bonner (1947) found considerable loss of phosphatase activity, so partial modification of the proteins seems likely.

Acid precipitation has been found by some workers to lead to denaturation (Chibnall, 1939; Wildman & Gordon, 1942), but under conditions used here reasonably good recoveries have been obtained. The best recoveries of total soluble nitrogen and phosphorus, as well as of the more labile components of the extract, resulted from ethanol fractionation at low temperature. Phosphatase activity was also preserved, so little modification of the proteins seems to have occurred. Under the conditions used here, the separation of the main fractions was not as great as with salt precipitation, but the flexibility of the method, with independent variability of ionic strength, pH and ethanol concentration may enable improvements to be made in this respect.

Despite their similarity of appearance in the ultracentrifuge, the components of clover and ryegrass extracts showed considerable difference in solubility. In general, the ryegrass proteins were less readily precipitated; this is seen from Tables 2, 3 and 4. This higher degree of solubility may be related to the higher charge on the ryegrass proteins, which is evident from the higher electrophoretic mobility.

SUMMARY

1. The soluble cytoplasmic proteins extracted from the leaf cells of white clover and Italian ryegrass have been studied by electrophoresis and ultracentrifuging.

2. These extracts have been fractionated by high-speed centrifuging, salt precipitation, acid precipitation and ethanol precipitation.

3. A protein fraction which was monodisperse on electrophoresis and in the ultracentrifuge has been isolated from clover leaf, and its molecular weight established as approximately 600 000.

4. Close similarity is found to the types of protein reported in extracts from other plants. However, the monodisperse fraction from clover differs from that described from spinach and tobacco in that it is not associated with ribonucleic acid.

5. Leaves of both monocotyledonous and dicotyledonous plants appear to contain the same types of soluble cytoplasmic protein.

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The Metabolism of δ-Aminolaevulic Acid

1. NORMAL PATHWAYS, STUDIED WITH THE AID OF ^{15}N

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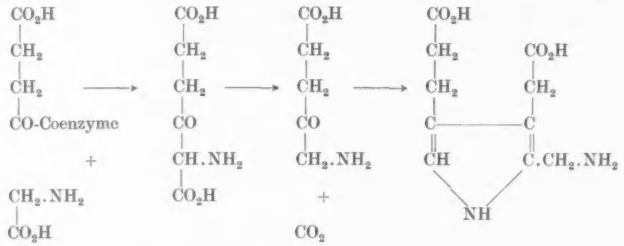
On the basis of results obtained with *in vitro* systems, δ-aminolaevulic acid (ALA) has been found by several groups of workers to be a biological precursor of porphyrins and haem. The following reactions are believed to occur (Shemin & Russell, 1953; Neuberger & Scott, 1953):



Shemin & Russell (1953) and Shemin, Russell & Abramsky (1955) have demonstrated that ALA is used in preference to glycine and succinate for haem synthesis by avian red cells. Shemin, Abramsky & Russell (1954) showed that a soluble enzyme system, obtained by centrifuging lysed avian red cells, would convert ALA into haemin. Gibson, Neuberger & Scott (1954, 1955) have isolated a widely distributed enzyme which catalyses the conversion of ALA into PBG.

The last compound, porphobilinogen (PBG), has been shown by Falk, Dresel & Rimington (1953) to be converted enzymically into porphyrins *in vitro*. Employing isotopically labelled glycine or ALA, Net synthesis of haem or porphyrin from glycine in a lysed avian red-cell system is very slight, but large quantities of porphyrins are formed when PBG is added in place of glycine (Falk *et al.* 1953). ALA also was found to give rise to free porphyrins under these conditions (Neuberger & Scott, 1953; Dresel & Falk, 1953), and was shown by the latter

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authors to be equivalent to PBG in this respect. At the same time, however, Neuberger & Scott stated that when [^{15}N]ALA was tested *in vivo*, its incorporation into the haem of circulating haemoglobin was much less than that resulting from an equivalent dose of [^{15}N]glycine. This finding is now reported in full, together with the results of subsequent studies *in vivo*. The experiments were designed to throw light on this low incorporation of ALA into haem, and also to investigate the overall metabolism of ALA in the intact animal. Preliminary accounts of some of these studies have already appeared (Berlin, Neuberger & Scott, 1954; Berlin, Gray, Neuberger & Scott, 1954; Scott, 1955).

Shemin & Russell (1953) postulated a succinate–glycine cycle (see also Shemin, 1955) involving ALA, and have indeed obtained some evidence for its existence. Operation of this postulated cycle could explain many of the metabolic inter-relationships involving glycine, and would place ALA, at least qualitatively, in a rather central position in intermediary metabolism. Quantitative aspects of the metabolism of ALA in intact animals acquire therefore an enhanced importance.

EXPERIMENTAL

Animals. Albino rats of the National Institute for Medical Research strain were employed. For preparation of haemin, the animals were killed 6 days after injection of [^{15}N]ALA.

Urine flow. Whenever urinary ALA and PBG were to be determined, physiological saline (2 ml.) was injected into each rat every 2 hr. for 8 hr., in order to overcome an apparent antidiuretic effect of ALA. For the same reason, human subjects were given 100 ml. of water hourly for 8 hr. after swallowing a solution of ALA.

Administration of δ-aminolaevulic acid. The [^{15}N]ALA (Neuberger & Scott, 1954) contained 30·6 atoms % excess ^{15}N. Immediately before injection, a solution of ALA hydrochloride was mixed with one equivalent of NaOH. Solutions of ALA allowed to stand after neutralization give a positive test with Ehrlich's reagent. For addition of ALA to the diet, a solution of the unneutralized hydrochloride was used. In the tests with human subjects, ALA was given orally as the hydrochloride dissolved in 200 ml. of water.

Glutathione (GSH) and ascorbic acid. Immediately before injection of these compounds they were dissolved in one equivalent of 0·2 N-NaOH.

Fractionation of urinary nitrogen

It was necessary to treat small urine samples, produced by rats which had received [^{15}N]ALA, in such a way that simultaneous values for the percentage excess of ^{15}N in the free NH_3, urea and residual N, together with the overall amounts of nitrogen (i.e. $^{14}N + {}^{15}N$) in these three fractions, could be obtained. The principle of the first method employed (method *A*) was to determine the total N, and its mean percentage of excess ^{15}N after Kjeldahl digestion and distillation of a measured fraction of urine. The remainder of the urine sample was then treated as described below to yield comparable figures for the free ammonia N and for the sum of free ammonia N and urea N. The values for the urea N and for the residual N were then calculated by difference, since the addition of urease in the last operation precludes a subsequent direct determination of residual N. All values reported (Fig. 1 and Tables 3–6) represent the means of complete determinations of N and of ^{15}N carried out in triplicate. Tests, using a mixture of known quantities of NH_4Cl, urea and ALA at concentrations similar to those found in the urine samples, indicated that recovery of the N in each fraction was not less than 96% with method *A*.

Total nitrogen. Two measured fractions of the urine sample, each equivalent to about 1 mg. of N, were digested for 24 hr. with 1·5 ml. of conc. H_2SO_4 containing SeO_2, $HgSO_4$ and K_2SO_4. One sample was used for determination of the excess of ^{15}N by the procedure of Sprinson & Rittenberg (1949). The other sample was distilled from a Kjeldahl apparatus into a known volume of 0·02 N-HCl; N was then estimated by back-titration with NaOH, Tashira's indicator (Cole, 1933) being used.

Ammonia nitrogen (method A). The remainder of the urine sample was divided accurately into two parts. The smaller part (one-fifth) was set aside for estimation of urea N. The larger part was transferred to a Kjeldahl flask and a saturated aqueous solution of NaOH (0·25 vol.) was added. Air or nitrogen was then bubbled through the mixture for 2 hr. at room temperature. Ammonia was trapped and treated as before, except that a known volume of the distillate was withdrawn for titration, the remainder being used for ^{15}N determination.

Ammonia plus urea nitrogen. The pH of the smaller fraction mentioned above was adjusted to neutrality, if necessary, and the urine was transferred to a Kjeldahl flask. The urea was then decomposed with urease (British Drug Houses Ltd.), added in tablet form. After standing for 2 hr., a saturated aqueous solution of NaOH (0·25 vol.) was added and the resulting ammonia was collected and treated as described for ammonia N (method *A*).

Other methods for isolation of nitrogenous constituents of urine. For reasons which are made clear when the Results are described, it was necessary to see whether the strong alkali used in the procedure described was causing any redistribution of ^{15}N among the three fractions referred to. For this purpose two procedures were developed; in both, the free NH_3 was removed without the use of strong alkali, leaving a solution from which the urea N could then be isolated directly. Since only the excess of ^{15}N in each fraction was to be measured, the fact that neither of these procedures permitted such accurate recoveries as did the original was immaterial.

Method B *for urine* NH_3. This method was elaborated from the data given by Folin & Bell (1917). A column was prepared in the following manner: dry Decalso F (Permutit Co., Gunnersbury Avenue, London, W. 4; 2 g. for 10 ml., or less, of diluted urine) was placed in a glass tube having an H3 sintered filter disk at the lower end; distilled water was then added, air bubbles were removed, and most of the water was allowed to run through under gravity. The urine sample was diluted with water (1 vol.) and the pH was adjusted to between 6 and 7 if necessary; the diluted urine was then added to dry Decalso F (0·2 g./ml. of diluted urine) in a small flask, which was agitated gently for 0·5 hr. The resulting slurry was poured on to the column, and washed in with water (1 vol.), the flow of liquid out of the tube being

limited throughout all the operations to just below 3 ml./min. When all the liquid had been run on, the column was washed with water (1 vol.). The column effluent was set aside for isolation of urea N as described below. The absorbed ammonia was then displaced from the column by elution with a saturated aqueous solution of KCl (2 vol.). Sufficient of the KCl eluate was then transferred to a Kjeldahl flask, and the excess of ^{15}N was determined as before.

Method C for urine NH_3. The procedure was essentially similar to that of Van Slyke (1915). The pH of the urine sample was adjusted to 3 with HCl, and an equal volume of 20% (w/v) phosphotungstic acid was added. The precipitated ammonium salt was separated by centrifuging, and washed by resuspension and centrifuging in 1% (w/v) phosphotungstic acid (2 × 10 ml.). The supernatant fluid left after the first separation was extracted with 50% (v/v) 'amyl alcohol' in ether until small samples of the aqueous layer no longer gave any precipitate with NH_3. After one further extraction with ether to remove the amyl alcohol, air was blown through the solution to remove the ether. This solution was set aside for isolation of urea N. Ammonia N and excess of ^{15}N were then determined by method *A*, the whole of the washed ammonium phosphotungstate precipitate being used in place of a measured fraction of a urine sample.

Urea nitrogen (*methods* B *and* C). A measured fraction of the column effluent (method *B*) or of the supernatant solution (method *C*) was treated with urease as described in method *A*.

Hippuric acid. This was isolated by the method of Arnstein & Neuberger (1951).

ALA. This was estimated by the picrate method of Shuster (1956); the results were frequently checked by his acetoacetate method.

Porphobilinogen. PBG was estimated by a modification of the method of Cookson & Rimington (1954). Before reaction with Ehrlich's reagent, the urine was so diluted that the optical density at 552 mμ. was less than 0·4 after addition of the reagent. In the human experiments, four drops of a saturated solution of $CuSO_4$ were added to every 10 ml. of diluted urine. This treatment was found to stabilize the colour subsequently obtained, particularly when the fading of the colour was due to the presence of thiols. PBG was isolated from human urine by the method of Cookson & Rimington (1954). The identity of the crystalline material so obtained was checked chromatographically, with the system described below. It was applied to the paper as a solution in dilute ammonia.

Chromatographic separation of urine constituents. Rat urine (10 ml.) was freeze-dried after adjusting to pH 2 with HCl. The residue was then extracted with 2 ml. of 90% (v/v) ethanol, and the ethanolic solution was applied to Whatman no. 1 paper. The chromatogram was run for 16 hr. in *n*-butanol–water–acetic acid (63:27:10 by vol.) at 25°; this is similar to the solvent system employed by Cookson & Rimington (1954). Under our conditions the following R_F values were observed: glycine 0·13, ALA 0·23, PBG 0·48. The first two compounds were located by spraying with ninhydrin (0·1% solution in *n*-butanol) and drying at 60°. Under these conditions ALA gives a brown–yellow spot, which later turns purple. PBG was located by spraying with Ehrlich's reagent.

Faecal nitrogen

The combined 24 hr. faeces from two rats were digested with 20 ml. of conc. H_2SO_4 and catalysts referred to under 'Total N' above. When digestion was complete (3–4 days) the acid solution was cooled and made up to a known volume. For total faecal N, 0·02 vol. was removed and treated as described above.

Amide nitrogen from liver protein

The washed liver was ground in a mortar with 80% (v/v) ethanol; the suspension was boiled and filtered. The residue was washed by centrifuging in ethanol and then in ether, and dried. The precipitate (1·0 g.) was then boiled under reflux for 3 hr. with 2·5 N-HCl (25 ml.). On cooling, the solution was made alkaline to phenolphthalein with $Ba(OH)_2$; the ammonia thus liberated was then distilled *in vacuo* at room temperature into an excess of 2 N-H_2SO_4. This solution was then transferred to a Kjeldahl flask, made strongly alkaline with 10 N-NaOH, and distilled with heating into an excess of HCl for subsequent determination of ^{15}N.

RESULTS

Incorporation of ALA into haem

The incorporation into haem of isotope from different labelled compounds may be satisfactorily compared by calculating the 'corrected dilution coefficient', C.D.C. (Arnstein & Neuberger, 1951). This was defined by the relationship

$$\text{C.D.C.} = Q(C_Q/C - 1),$$

Table 1. *Incorporation of* [^{15}N]*ALA or* [^{15}N]*glycine into haem*

The [^{15}N]ALA containing 30·6 atoms % excess ^{15}N was either given in the diet or injected intraperitoneally (IP), as described in the text. GSH (rat 34) or ascorbic acid (rat 38), at a dose level of 0·2 m-mole/100 g., was injected simultaneously with the [^{15}N]ALA. For definition of 'corrected dilution coefficient' (C.D.C.) see text.

Rat (nos.)	Dose of ALA (m-mole/100 g.)	Route of administration	% excess ^{15}N in haem	C.D.C.
19 + 20	0·20	Fed	0·013	466
17 + 18	0·20	IP	0·018	340
34	0·20 (+ GSH)	IP	0·017	360
36	0·20 (+ ascorbic acid)	IP	0·017	360
30 + 31	0·50	IP	0·045	339
12	Glycine 0·20	Fed	0·105	60*

* Calculated from the data of Muir & Neuberger (1949).

Table 2. *Utilization of ALA-N for hippuric acid and urea synthesis*

In the experiments in which hippuric acid was isolated, each animal received 0·20 m-mole of sodium benzoate/100 g. at the same time as the labelled compound. Abbreviations are the same as in Table 1.

Rat (nos.)	Substance administered: [^{15}N]ALA (m-mole/100 g.)	Substance administered: [^{15}N]glycine (m-mole/100 g.)	% excess ^{15}N	% excess ^{15}N in hippuric acid	% excess ^{15}N in urea	C.D.C.
21 + 22	0·165 (IP)	—	30·6	0·075	—	67·1
*	—	0·033 (IP)	15·5	1·278	—	0·366
19 + 20	0·160 (fed)	—	30·6	—	0·042	117
17 + 18	0·160 (IP)	—	30·6	—	0·044	111
23	—	0·160 (IP)	31·4	—	0·047	106

* Data of Arnstein & Neuberger (1951).

where Q is the dose (m-moles/100 g. body weight) and C_0 and C are the isotope concentrations of the labelled atoms in the starting material and in the product isolated, respectively. C.D.C. is expressed in the same units as the dose. Since glycine N or ALA nitrogen is the sole source of haem N, this equation required no modification to take into account the fact that more than one glycine molecule is required in order to form one haem molecule.

Table 1 shows that [^{15}N]glycine was incorporated 5 to 6 times as readily as [^{15}N]ALA in the intact rat. Slightly less was incorporated when ALA was fed, rather than injected, owing presumably to the ready oxidation of neutral solutions of ALA exposed to the air. Considered alone, these findings would have suggested that ALA was behaving more as a precursor of glycine than as a porphyrin precursor. This possibility was eliminated by isolating hippuric acid formed from benzoate administered either with [^{15}N]glycine or with [^{15}N]ALA. The ^{15}N content of the hippuric acid formed from [^{15}N]ALA was of the same order as that of the urea isolated in a parallel experiment. [^{15}N]Glycine, while it gave rise almost to the same labelling in the urea as [^{15}N]ALA, was used for hippuric acid synthesis to a very much higher degree (Table 2).

Urinary excretion of ^{15}N

Attention was next turned to the distribution of isotope amongst the urinary constituents. It was found that from one-third to two-thirds of the isotope administered as [^{15}N]ALA was eliminated by this route, and that the bulk of the ^{15}N was in the 'residual' urinary nitrogen, that is the fraction remaining after free ammonia and urea had been removed (Fig. 1). The excretion was rapid, since 75 % of the ^{15}N excreted in this way during the first day had already been eliminated in the first 6 hr. When glutathione (GSH) or ascorbic acid was injected simultaneously with [^{15}N]ALA, an increased ^{15}N excretion was observed, while the distribution of isotope in the three fractions of urinary nitrogen was little altered (Table 3). This increase cannot, however, be considered significant as subsequent experiments, discussed below, have shown. The reasons for injecting GSH or ascorbic acid are explained below. Neither GSH nor ascorbic acid increased the incorporation of ALA into haem (Table 1). The isotope content of liver-protein amide nitrogen, relative to the size of the dose, was higher in rat 36, which had ascorbic acid, than in rats 30 + 31 (Table 3).

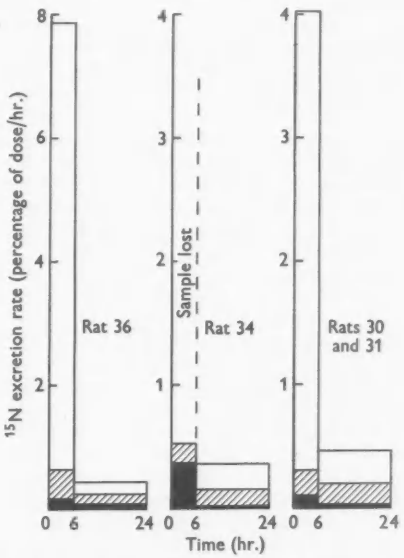

Fig. 1. Rates of urinary excretion of ^{15}N by rats which had received [^{15}N]ALA. The rats were injected intraperitoneally with a neutralized solution of [^{15}N]ALA hydrochloride, containing 30·6 atoms % excess ^{15}N. The amounts of [^{15}N]ALA received were as follows: rats 30 + 31, 0·125 m-moles/100 g.; rats 34 and 36, 0·05 m-mole/100 g. ■, $NH_3{}^{15}N$; ▨, urea ^{15}N; □, residual ^{15}N.

It may be seen from Table 4 that the percentage of excess ^{15}N in the ammonia was extremely high, and was still apparently greater than that in the urea on the day after the last injection of [^{15}N]ALA. Previous work in which ^{15}N-labelled L-amino acids or $^{15}NH_4Cl$ (see Discussion) have been administered has shown that little of the isotope is found in the urinary free ammonia, and has indicated that free ammonia in the body is rapidly converted into urea. Wu (1950) pointed out that the ^{15}N concentration in the ammonia and the urea excreted during such experiments was time-dependent, but that the ratio of percentage excess ^{15}N in the NH_3 and urea had generally fallen below unity after a few hours. In view of these well established results, discussed more fully below, we therefore subjected our own findings with [^{15}N]ALA to a closer scrutiny. It was necessary to establish that the NH_3 isolated by method *A* did in fact represent the original urinary free NH_3, and that its ^{15}N content had not been increased during isolation by exchange with another urinary constituent, such as ALA, which would be expected to have a high ^{15}N content. Similar experiments were therefore performed, after which the urinary free NH_3 was isolated either by adsorption on Decalso F (method *B*) or by precipitation with phosphotungstic acid (method *C*). The results of these experiments, shown in Table 5, fully substantiate those of Table 4. By dilution with $^{14}NH_4Cl$ it was found, in experiments not reported here, that the [^{15}N]ALA contained a small amount of $^{15}NH_4Cl$ (approximately 5%, w/w), which resisted removal by repeated recrystallization from dry methanol–ether and dry methanol–ethyl acetate. On injection, this impurity would be converted rapidly into urea, as shown by Schoenheimer (1946), and would not therefore give rise to a high ^{15}N concentration in the free urinary NH_3 many hours after administration.

Nature of 'residual' nitrogen

The ^{15}N excretion data showed that the largest contribution was from the 'residual' fraction of the urine N; components of this fraction were therefore sought. It was believed that a major component would be unchanged ALA, but the first component identified and isolated was PBG (Berlin, Neuberger & Scott, 1954). Our finding that PBG is excreted after injection of ALA has recently been confirmed (Shemin, Gatt, Schmid & Weliky, 1955).

Porphobilinogen. The amount of metabolized ALA excreted as PBG reaches a maximum in the rat at a dose level of about 0·5 m-mole of ALA/100 g.; the absolute amount excreted also reaches a maximum at about this value, indicating saturation of the capacity of the organism for converting ALA into PBG under these conditions. This is illustrated in Fig. 2 (see also Table 8). Since completion of the experiments summarized in Fig. 2,

Table 3. *Urinary ^{15}N excretion pattern of rats which received [^{15}N]ALA*

In each case the [^{15}N]ALA was injected intraperitoneally in four equal parts on successive days. The values for ^{15}N content of haemin prepared from these rats are shown in Table 1. Rats 30 + 31, 34 and 36 weighed 491, 240 and 244 g. respectively. Amide N was obtained from liver protein as described. NH_3 was isolated by method *A*.

Rat (nos.)	Compound injected (m-moles/100 g.) ALA	Other compounds	Urine N component	Totals after 1 day: $^{14}N + ^{15}N$ (mg./100 g.)	% excess ^{15}N	% of 1-day dose ^{15}N	% of total urine excess ^{15}N	Totals after 5 days: % of total dose ^{15}N	% of total urine excess ^{15}N	Amide ^{15}N in liver protein (% excess) after 6 days
30 + 31	0·50	None	Urea	52·2	0·048	4·4	12·9	4·0	13·6	0·017
			NH_3	2·74	0·226	1·1	3·2	1·3	4·5	
			Residual	13·8	1·18	28·4	83·8	24·2	81·9	
			Total (determined)	68·7	0·283	33·9	—	29·6	—	
34	0·20	0·20 GSH	Urea	49·0	0·012	2·6	?	4·5	10·1	Not determined
			NH_3	2·73	0·222	2·6	?	2·0	4·5	
			Residual	Not calculable				37·9	85·2	
			Total (determined)	Lost				44·5	—	
36	0·20	0·20 ascorbic acid	Urea	90·5	0·016	6·3	11·1	6·9	13·4	0·017
			NH_3	4·15	0·068	1·2	2·2	2·2	4·3	
			Residual	10·5	1·08	49·4	86·5	42·5	82·3	
			Total (determined)	105	0·124	57·0	—	51·6	—	

Table 4. *Ratios of* ^{15}N *content and* ^{15}N *concentration in urea and* NH_3 *(by method* A)

The total dose given to each rat is shown in Table 3; it was given in four equal parts on four successive days. Ammonia N was isolated and determined by method *A*.

Days after start of experiment	Rats 30+31: Total excess urea ^{15}N (mg.)/ total excess NH_3 ^{15}N (mg.)	Rats 30+31: NH_3 ^{15}N % excess/ urea ^{15}N % excess	Rat 34: Total excess urea ^{15}N (mg.)/ total excess NH_3 ^{15}N (mg.)	Rat 34: NH_3 ^{15}N % excess/ urea ^{15}N % excess	Rat 36: Total excess urea ^{15}N (mg.)/ total excess NH_3 ^{15}N (mg.)	Rat 36: NH_3 ^{15}N % excess/ urea ^{15}N % excess
0·25	2·1	8·2	0·4	27·4	3·0	8·7
1	6·7	2·9	7·1	3·0	11·1	1·9
2	2·2	8·3	1·1	8·2	2·1	8·6
3	2·7	4·8	5·8	5·6	2·1	10·3
4	2·9	5·0	1·2	10·4	5·2	2·8
5	7·4	4·2	2·8	9·9	11·1	2·0

Table 5. *Concentration of* ^{15}N *in urea and* NH_3 *determined by method* B *or* C

[^{15}N]ALA was injected intraperitoneally in a single dose. Free urinary NH_3 was isolated by adsorption on to Decalso F (method *B*) or by precipitation with phosphotungstic acid (method *C*). Values for ^{15}N percentage excess below 0·005 were not considered significant when the ratios in the final column were calculated.

Rat (nos.)	Dose of ALA (m-mole/100 g.)	Period of urine collection (hr.)	Method of isolation	NH_3 ^{15}N (% excess)	Urea ^{15}N (% excess)	NH_3 ^{15}N % excess/urea ^{15}N % excess
49+50	0·05	0–24	*B*	0·386	0·050	7·5
		24–48	*B*	0·010	0·004	>2
51	0·15	0–24	*C*	0·928	0·158	5·9
		24–48	*C*	0·010	0·002	>2
54	0·15	0–24	*C*	0·854	0·157	5·4
		24–48	*C*	0·009	0·006	1·5

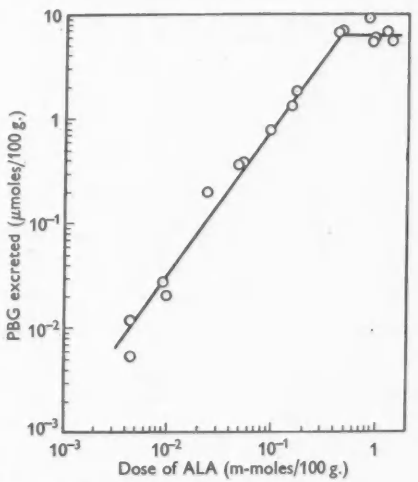

Fig. 2. Relationship between the dose of ALA and the amount of urinary PBG excreted by rats. Both axes are measured on a logarithmic scale. No rat was used more than once, and the body weights were all within the range 180–250 g.

we have found that addition of Cu^{2+} ions to the urine before reaction with Ehrlich's reagent prevents the colour from fading (see Experimental section). With a stable Ehrlich colour, the optical density may be as much as 10% higher; the percentage of ALA converted into PBG was therefore somewhat greater than would appear from Fig. 2.

δ-Aminolaevulic acid. Urine voided in the first 6 hr. after injection of ALA was subjected to chromatography as described. After spraying with ninhydrin solution there appeared, in addition to the usual amino acid spots, a large yellow spot (R_F 0·24) which was found to be unchanged ALA by comparison with the pure compound. This procedure, while indicating that a large quantity of unchanged ALA was being excreted, was not, however, suitable for estimation. ALA was later determined by methods developed by Shuster (1956). As shown in Table 6, the excretion of ALA was sufficiently great to account for most of the residual N fraction, assuming that the [^{15}N]ALA excreted was not greatly diluted by endogenous ALA. Proof of the validity of this assumption is supplied in the next paper (Berlin, Neuberger & Scott, 1956).

Other components. No gross increase in free urinary porphyrins was detected after administration of [^{15}N]ALA. The urine was darker than normal, and became dark brown on standing, but no pink fluorescence was observed in ultraviolet light. The pigment could not be extracted into organic solvents at any pH. The absorption spectrum of this urine, with a band at 490–496 mμ., which sharpened without change in position after acidification of the urine with HCl, suggested the presence either of 'porphobilin' (Waldenström & Vahlquist, 1939; Prunty, 1945) or of the mixture of pigments formed from PBG and described by Brockman & Gray (1953).

Excretion of ALA and PBG

In view of the apparent increase in aminolaevulic-dehydrase activity found in the livers of rabbits poisoned by repeated doses of Sedormid (Gibson *et al.* 1955), it was of interest to see whether repeated doses of ALA would provoke any change in the amounts of ALA and PBG excreted. Gibson *et al.* (1954) also described the activation of ALA dehydrase by GSH and tests were therefore made to ascertain whether the enzyme was further activated in the body by injecting GSH at the same time as large doses of ALA. Results of these experiments are summarized in Table 6. Repeated daily injections of ALA had no demonstrable effect on the amounts of ALA and PBG subsequently excreted. When GSH was injected at the same time as [^{15}N]ALA, the amount of residual ^{15}N appeared to be increased (Table 3). But this observed increase cannot be taken as significant in view of the considerable variations found among animals not so treated (Table 6). The percentage of ALA excreted unchanged was not higher when GSH was given simultaneously than the upper value observed after injection of the same quantity of ALA alone; nor was the amount of PBG excreted significantly increased by administration of GSH.

Attempts were made to determine and isolate ALA-N in urine for ^{15}N analysis by oxidation with periodate, a method which has also been described by Shemin, Russell & Abramsky (1955). A 95–100 % recovery was obtained when the oxidation was carried out with pure ALA, but the method was found to be unsatisfactory with urine, owing to the presence of other substances which evolve NH_3 under these conditions.

Faecal excretion of ALA nitrogen

The total faecal N and ^{15}N after injection of [^{15}N]ALA is shown in Table 7, the total urinary N and ^{15}N excreted during the same period are also given, since the ALA excreted has been shown to vary between such wide limits (Table 6).

Table 6. *Effect of repeated doses of ALA and of GSH on the excretion of ALA and PBG*

Rat (nos.)	Days after start of experiment	Compound administered intraperitoneally (m-mole/100 g.)		Percentage of daily dose of ALA excreted in 24 hr. in the form indicated		
		ALA	GSH	ALA	PBG	Total
101 + 102	1	0·20	—	37·7	3·0	40·7
	2	0·20	—	60·0	2·4	62·4
	3	0·20	—	56·7	2·5	59·2
	4	0·20	—	61·1	2·9	64·0
	5	0·20	0·20	45·7	3·0	48·7
103 + 104	1	0·20	0·20	58·4	2·8	61·2
105 + 106	1	—	0·20	—	—	—
	2	0·20	0·20	44·9	3·3	48·2

Table 7. *Faecal and urinary excretion of ALA nitrogen*

Rats 52 + 53, of total weight 406 g., were given 0·15 m-mole of [^{15}N]ALA (30·6 atoms % excess)/100 g. by intraperitoneal injection. The urine and faeces were separated on voiding.

Time after injection (hr.)	Faecal N (mg.)	Faecal ^{15}N (% excess)	Percentage of the dose of ^{15}N	Total percentage excreted
24	123	0·161	7·6	—
48	103	0·090	3·6	11·2
	Urine N (mg.)	Urine ^{15}N (% excess)		
24	434	0·431	70·2	—
48	504	0·013	2·5	72·7
				83·9

Effects of ALA in man

Urinary excretion products. The rates of excretion of ALA and PBG in the urine, after ingestion of ALA by a human subject, are shown in Fig. 3. There was little variation ($< \pm 1\%$) between different subjects at the same dose level. The PBG was isolated from the urine and crystallized. It was chromatographically indistinguishable from PBG isolated similarly from the urine of an acute porphyric.

As in the rat, the percentage of the dose converted into PBG increased with the dose within the range tested (Table 8), indicating an increasing saturation of aminolaevulic dehydrase in the tissues concerned.

Photosensitization. Administration of ALA to man was followed, in all normal subjects tested, by an erythema of the exposed skin. The severity of the

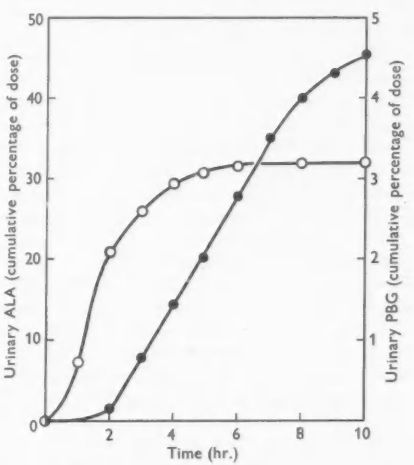

Fig. 3. Excretion of ALA and PBG by a human subject after ingestion of ALA. This subject (B.A.A.) received 0·0095 m-mole of ALA/100 g., and excreted 4·6% of the dose as PBG in 24 hr. ○, ALA (left-hand axis of ordinates); ●, PBG (right-hand axis of ordinates). Similar curves were obtained by plotting the data from other subjects (Table 8).

Table 8. *Percentage of administered ALA excreted as urinary PBG in human subjects*

Subject	Dose (m-moles/100 g.)	Percentage of the dose excreted as PBG
J.J.S.	0·0075	4·76
J.J.S.	0·0075	4·10
N.I.B.	0·0091	3·29
B.A.A.	0·0095	4·62
J.J.S.	0·0150	6·76
K.D.G.	0·0210	8·54

Table 9. *Photosensitivity following ingestion of ALA by human subjects*

Subject	Sex	Dose (m-moles/100 g.)	Appearance of erythema (hr. after dose)	Date	Weather	Exposure to sun	Nature of symptoms	Duration of symptoms (days after dose)
J.J.S.	m	0·0075	10	29. iii. 54	Foggy	None	Erythema and tingling sensation (face); eyes smarting and bloodshot (sunglasses not worn)	1
J.J.S.	m	0·0075	11	11. v. 54	Cloudy	None	Erythema and tingling sensation (face)	1
J.J.S.	m	0·0150	8	14. iv. 54	Cloudy	None	Erythema and burning sensation (face and back of hands)	2
N.I.B.	m	0·0091	10	22. iv. 54	Cloudy	None	Erythema and burning sensation (face)	1
B.A.A.	f	0·0095	7	24. vi. 54	Sunny	20 min. in sun after 5 hr.	Erythema (face and hands), replaced after the first day by suntan	1 30
K.D.G.	m	0·0210	2	25. ii. 55	Sunny with snow on ground	7 min. in sun after 5 hr.	Erythema, with severe and painful burning (face only) lasting 4–24 hr. Exfoliative eruption all over face Desquamation Recovery	1 1–2 3–10 21

response increased with the dose, but variation in response between subjects appeared to be considerable; this may have been due in part to the variation of light intensity to which the subjects were exposed. Except during the first test, sun-glasses were worn throughout the day; this is an advisable precaution. The preliminary findings are reported in Table 9, pending a fuller investigation of this phenomenon.

DISCUSSION

Excretion pattern of aminolaevulic acid nitrogen

After administration of [^{15}N]aminolaevulic acid to rats a proportion of the labelled nitrogen, which varied between 30 and 70%, was excreted in the urine within 24 hr. after administration (Tables 3 and 7). Between 75 and 87% of the urinary ^{15}N was found in the residual fraction, i.e. the fraction which remains after removal of urea and ammonia. These findings are in striking contrast with those obtained with ^{15}N-labelled L-α-amino acids, where it was found that, with doses similar to those used here, 25–40% of the labelled nitrogen was excreted by 25 or 48 hr. Moreover, 90% of the ^{15}N excreted was in the form of urea (Schoenheimer, Ratner & Rittenberg, 1939*a*; Ratner, Rittenberg, Keston & Schoenheimer, 1940; Weissman & Schoenheimer, 1941). With α-amino acids only about 3–6% of the administered ^{15}N appeared in the faeces. The larger proportion observed after giving aminolaevulic acid (Table 7) is probably due to the presence of labelled bile pigment and porphyrins (Berlin *et al.* 1956).

A small part, probably 5% (Table 6), of the ^{15}N of the residual nitrogen fraction is due to porphobilinogen, but most of it is in the form of unchanged aminolaevulic acid. This deduction is based on the close similarity in the order of values of the proportion of the dose excreted in the urine and measured colorimetrically as aminolaevulic acid (Table 6) and that of ^{15}N present in the residual fraction of the urinary nitrogen (Table 3). A similar parallelism was observed in the time relationship of the excretion of ^{15}N and of that of aminolaevulic acid; by either criterion it was found that most of the excretion occurred in the first 6 hr. (Fig. 1), and almost none was excreted after 24 hr. (Fig. 3).

Another unusual finding is the very high ratio of isotope content (in terms of atom excess ^{15}N) of the urinary ammonia to that of urea (Table 4). With almost all L-amino acids this ratio was found to be 1·2–1·5 during the first few hours after administration, to decrease later to 1·0 or slightly below 1·0 (for review see Wu, 1950). With L-aspartic acid an initial value of 0·55 has been observed (Wu & Rittenberg, 1949). The values found for this ratio with aminolaevulic acid (Tables 4 and 5) were almost always appreciably higher than 1·0, even many hours after administration, were frequently of the order of 8–11, and in one experiment a figure of 27·4 was obtained. These high values cannot be explained by contamination of [^{15}N]aminolaevulic acid with $^{15}NH_4Cl$, since ammonium salts are greatly inferior to L-amino acids as precursors of urinary ammonia; for the rat, the rabbit and man, ratios of 0·46 (Schoenheimer, 1946), 0·19 (Waelsch & Rittenberg, 1942) and 0·15 (Sprinson & Rittenberg, 1949) respectively have been reported. It is also most unlikely that the high ^{15}N content of the ammonia fraction results from chemical manipulations involved in the isolation procedure, since three different methods yielded similar high values (Table 5). It thus follows either that aminolaevulic acid is deaminated in the kidney and is, like glutamine, a specific precursor of urinary ammonia, or that ammonia is removed from aminolaevulic acid or porphobilinogen or some other metabolite of aminolaevulic acid after the labelled substance has left the kidney tubules.

These findings with aminolaevulic acid resemble closely those obtained with racemic or D-[^{15}N]amino acids. Thus with DL-tyrosine (Schoenheimer, Ratner & Rittenberg, 1939*b*), D-leucine (Ratner, Schoenheimer & Rittenberg, 1939) and D-lysine (Ratner, Weissman & Schoenheimer, 1943) 60–70% of the administered ^{15}N was excreted within 24 hr., and a large proportion of the urinary ^{15}N was found in the residual nitrogen fraction. The ratio of isotope content of the ammonia fraction to that of the urea varied with the D-amino acids mentioned between 4 and 6, but with DL-serine a ratio of 10 was found (Stetten, 1942). These findings have been explained by assuming that a large fraction of the D-amino acid is excreted as such, whilst another portion is deaminated by the D-amino acid oxidase of the kidney.

Tables 6 and 8 show the amounts of porphobilinogen excreted in the urine but, owing to the simultaneous formation of porphyrins and bile pigment (Berlin *et al.* 1956), the quantities formed must have been much greater. Granick & Vanden Schrieck (1955) reported the presence of a zinc porphyrin in the urine of a rat given aminolaevulic acid. In the present work no significant amounts of porphyrins or metallo-porphyrins could be detected in the urine.

Although a large proportion of the aminolaevulic acid was excreted as such or converted into pyrrolic metabolites, some of the nitrogen was used for the synthesis of amide groups of proteins (Table 3), the formation of urea (Tables 2 and 3) and the synthesis of amino acids (Table 2), indicating that an appreciable proportion of the nitrogen has become incorporated into the general 'nitrogen pool' of the body. This must arise, at least partly, from the liberation of ammonia occurring during the con-

version of porphobilinogen into porphyrins, but it may also be due to degradation. The possibility of a deamination of aminolaevulic acid itself has already been discussed above in relation to the ammonia results.

Incorporation of aminolaevulic acid nitrogen into haem

The rapid elimination in the urine of a large part of the aminolaevulic acid suggests that this substance is poorly, if at all, reabsorbed by the kidney tubules, resembling in this respect porphobilinogen (Goldberg & Rimington, 1954). The latter is assumed to be a necessary intermediate in haem synthesis; it would therefore appear likely that neither aminolaevulic acid nor porphobilinogen escapes from the cells of the haemopoietic system, since they are not normally excreted in significant amounts relative to the rate of haem synthesis.

The question next arises as to whether ALA or PBG can *enter* the cells of the haemopoietic system. Aminolaevulic acid N is incorporated into haem by the intact animal (Table 1). Comparison of the C.D.C. values shows that glycine is incorporated into haem 5–6 times as readily as aminolaevulic acid, but that aminolaevulic acid N gives rise practically to no glycine N as measured by hippuric acid (Table 2). The haem N labelling must therefore have come directly by penetration of aminolaevulic acid or porphobilinogen into the haemopoietic sites and not by a breakdown followed by non-specific re-utilization of its nitrogen. Immature red blood cells cannot therefore be completely impermeable both to ALA and to PBG. Further evidence on this point is brought forward in the subsequent paper (Berlin *et al.* 1956); it is convenient to defer further consideration of these results until they can be discussed in the light of the findings there described.

Experimental photosensitivity in man, induced by aminolaevulic acid

The marked photosensitivity of human subjects who have been given aminolaevulic acid is of particular interest in view of the severe photogenic blistering which is the main clinical feature of congenital porphyria. The nature of the reaction in the experimentally induced condition is, however, different from that usually observed in the disease, where exposure to light will have occurred over long periods. In none of the human subjects was there any vesicular eruption. Only in the subject who received the largest dose was there any desquamation; in all subjects the condition in fact resembled most closely that of sunburn. The photosensitivity of congenital porphyria has long been assumed to be due to porphyrins, particularly uroporphyrin I, known to be present in the blood and tissues of patients with this disease. Preliminary experiments have shown that protoporphyrin is excreted in relatively large quantities in the bile of rats after administration of ALA (Scott, 1955). Circulating porphyrins may therefore be responsible for the experimentally induced sensitivity; the skin reaction is, however, very mild in the latter condition, compared with that in the disease, where exposure with porphyrinaemia has been prolonged.

SUMMARY

1. [^{15}N]Aminolaevulic acid (ALA) was fed or administered parenterally to normal human subjects and to rats. Incorporation of ^{15}N into the haem of the circulating haemoglobin of rats was six to seven times less than was reported for an equivalent amount of [^{15}N]glycine.

2. Administration of [^{15}N]ALA produced labelling in the urinary urea to the same extent approximately as [^{15}N]glycine, but the latter was much more efficient as a precursor of hippuric acid than the former. [^{15}N]ALA also gave rise to significant labelling in the amide groups of liver proteins.

3. A large proportion of the ALA is excreted unchanged in the urine, both in man and in the rat within a few hours of administration. It is concluded that ALA is poorly, if at all, reabsorbed by the tubules of the kidney.

4. Most of the ^{15}N present in the urine was found in the fraction remaining after removal of urea and ammonia, and must be presumed to have been mainly in the form of unchanged ALA. In almost all experiments it was observed that the ^{15}N concentration in the urinary ammonia was considerably greater than in the urea, even after 2 days. The possibility of a specific deamination of ALA by the kidney is discussed, but alternative explanations, involving a post-renal release of labelled ammonia, have not been excluded.

5. Administration of ALA to man or rat has been found to give rise to excretion of appreciable amounts of porphobilinogen (PBG) in the urine. The conversion of ALA into PBG increased with the dose level up to a maximum.

6. Administration of ALA to human subjects produced skin reactions suggesting photosensitization. The bearing of this experimentally induced sensitivity on the explanation of the clinical condition associated with congenital porphyria is discussed.

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The Metabolism of δ-Aminolaevulic Acid

2. NORMAL PATHWAYS, STUDIED WITH THE AID OF ^{14}C

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In the preceding paper (Berlin, Neuberger & Scott, 1956) are described the results of investigations of the metabolism of δ-aminolaevulic acid (ALA) by administration of this compound to rats and to humans, and by studying the fate of the nitrogen of [^{15}N]ALA in rats. It was shown that a large proportion of the ALA was rapidly excreted as such, together with a small amount of porphobilinogen (PBG). The incorporation of isotope into haem was lower than expected, being less than that from an equivalent dose of [^{15}N]glycine.

In this paper is described a more detailed investigation into the fate of administered ALA. Because of the greater dilution detectable, and because it affords information about other parts of the molecule, [1:4-$^{14}C_2$]ALA was employed. The labelling was thus in the positions corresponding to the carboxyl carbon atoms of succinic acid, from which ALA is believed to be derived biologically.

The object of those experiments in which rats were used was twofold. It was desired to obtain data comparable with those resulting from our experiments with [^{15}N]ALA. It was also necessary to study the distribution of radioactivity from [1:4-$^{14}C_2$]ALA before administering it to a human subject.

There have already appeared preliminary accounts of parts of this work in which [1:4-$^{14}C_2$]ALA was administered to rats and to a human subject

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(Berlin, Neuberger & Scott, 1954; Berlin, Gray, Neuberger & Scott, 1954; Scott, 1955) and to cockerels (Neuberger, 1955).

EXPERIMENTAL

Preparation and isolation of compounds

^{14}C-Succinic acid. [1:4-$^{14}C_2$]Succinic acid (Radiochemical Centre, Amersham Bucks.), after dilution with non-isotopic succinic acid, contained 32·0 μc/m-mole.

δ-Aminolaevulic acid. [1:4-$^{14}C_2$]ALA was prepared by the method of Neuberger & Scott (1954), starting with [1:4-$^{14}C_2$]-succinic acid (Radiochemical Centre, Amersham, Bucks). The isotopic succinic acid was cyclized to the anhydride, in 94% yield, by refluxing with an excess of freshly distilled acetyl chloride. After evaporation to dryness *in vacuo* from a boiling-water bath, the crude anhydride was used for the subsequent reaction without purification. The crude hydrochloride of [1:4-$^{14}C_2$]ALA was recrystallized from cold dry methanol and dry ether; it had m.p. 147–149°, and contained 29·5 μc/m-mole.

ALA was administered intraperitoneally to rats and orally to human subjects by the methods described (Berlin *et al.* 1956). Estimations of ALA were carried out by both the picrate and acetoacetate methods of Shuster (1956).

Porphobilinogen. PBG was isolated by the method of Cookson & Rimington (1954). Determination was by the modified method described (Berlin *et al.* 1956).

Porphyrins and stercobilin. Stercobilin and crude porphyrins were isolated from the faeces by the methods used by Gray & Neuberger (1950) and by Gray, Neuberger & Sneath (1950) respectively. After separation by partition between ether and HCl (Zeile & Rau, 1937), coproporphyrin and protoporphyrin were estimated by measurement of the optical density at the peak of the Soret band in 0·1 N-HCl (Jope & O'Brien, 1945) and 2 N-HCl (Bois, 1927) respectively. The crude porphyrin, which consisted almost entirely of protoporphyrin, was then esterified and purified chromatographically on $CaCO_3$ by the method of Grinstein, Schwartz & Watson (1945). Before assay of radioactivity, stercobilin was recrystallized as the hydrochloride from chloroform–light petroleum (boiling range 60–80°), and protoporphyrin was recrystallized as the methyl ester from chloroform–methanol.

Blood haemoglobin. This was determined by the method of Wong (1928).

Apparatus and methods

Animals. Albino rats of the National Institute for Medical Research strain were used. After injection of labelled ALA, they were kept either in ordinary metabolism cages or else in a special metabolism jar of the type illustrated in Fig. 1.

Collection of CO_2 from rat breath. For collection of CO_2 from the breath, a rat was placed in the metabolism jar (Fig. 1), and a brisk stream of air was drawn through the apparatus. Food and water were provided, and coprophagy was not observed.

Collection of CO_2 from human breath and conversion into $BaCO_3$. One end of a length of glass tubing (1 cm. bore) was worked in a flame so that a flange was formed at right angles to the axis, and of about 3·5 cm. in diameter. The other end of the tube was connected, through a flutter valve followed by a large glass tap, to a meteorological balloon; a conventional Douglas bag was found to be too large, and could not easily be emptied quantitatively. Before giving a breath sample, the subject breathed into the balloon for about 5 min., breathing air in through the nose and expelling it through the glass tube, the flanged end of which fitted comfortably between the lips and the teeth. On each occasion, after a 5 min. practice period, the balloon was disconnected, rapidly emptied, reconnected, and a sample taken for precisely 1 min. The balloon was subsequently emptied and the CO_2 converted quantitatively into $BaCO_3$ by the method of Berlin, Tolbert & Lawrence (1951).

Blood ^{14}C. In the experiment with a human subject, blood samples (10 ml.) were withdrawn from an arm vein into a sterile syringe containing heparin, and were immediately centrifuged in graduated tubes, and the haematocrit value was noted. For each sample, plasma (0·50 ml.) and packed red cells (0·2–0·4 g. weighed accurately) were removed and dried *in vacuo* for combustion.

Total urinary and faecal ^{14}C. A measured volume (2–3 ml.) of each urine sample was transferred to a 100 ml. conical flask having a ground glass joint, and was dried in a vacuum desiccator. Each faeces sample was weighed and homogenized; a small quantity (about 0·5 g.) was weighed accurately in a tared conical flask and dried, and the dry weight was determined.

Combustion of total carbon to CO_2. Dried samples of blood, urine or faeces were combusted in the conical flasks with a

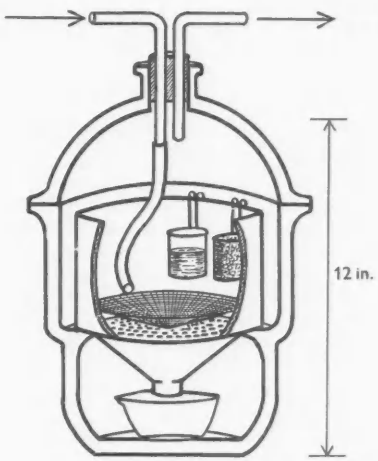

Fig. 1. Metabolism jar for collection of rat-breath CO_2. The jar consisted of a small desiccator (internal diameter, 9 in.) containing a Büchner funnel (internal diameter, 8 in.) with the spout cut short. The exposed unglazed porcelain was coated with silicone grease to prevent loss of urine. A shallow cone of wire gauze or perforated zinc was placed inside the funnel to prevent elution of the faeces by the urine, the latter being collected in an evaporating dish. The exit tube was led into a CO_2 trap (Berlin, Tolbert & Lawrence, 1951) and thence to a water pump.

chromic acid–sulphuric acid mixture (Van Slyke & Folch, 1940); the CO_2 evolved was collected and converted into $BaCO_3$ by the method of Berlin *et al.* (1951).

Assay of radioactivity

The particular assay method used is indicated in the tables where the data are shown. Values obtained by the different methods were directly comparable, since figures agreeing within 3% were obtained when a sample of hippuric acid having 0·1 μc/mg. was assayed by all four methods.

Method A. The more radioactive samples (counting rate over three times the background rate) were counted on 0·3 cm.^{2} Perspex disks, with an end-window counter. When only 3–6·5 mg. of material was available, the observed counting rate was extrapolated from a calibration curve to obtain the 'infinite' thickness rate.

Method B. Certain $BaCO_3$ samples were counted at 'infinite' thickness on 2 cm.^{2} polythene disks.

Method C. Less active samples, in the form of $BaCO_3$, were taken to the Radiation Laboratory, University of California, Berkeley, where determination of the radioactivity was kindly carried out for us by Dr Tolbert. The samples were converted into CO_2 and counted in a 100 ml. ionization chamber operating at atmospheric pressure. In an ionization chamber of this size the sensitivity is 0·02 disintegration/min./mg. of $BaCO_3$ (B. M. Tolbert & Elton M. Baker, to be published; cf. Baker, Tolbert & Marcus, 1955).

Method D. Haemin samples prepared from the blood of birds were converted into CO_2 by dry combustion and the radioactivity was measured in a gas counter at the National Institute for Medical Research (Bradley, Holloway & McFarlane, 1954). Combustion yielded 98–99% of the theoretical amount of CO_2.

RESULTS

Preliminary experiments with rats

[1:4-$^{14}C_2$]ALA was injected into two rats, which were then placed at once in metabolism jars suitable for the collection of breath CO_2 (Fig. 1). The doses of ALA were respectively lower (rat 1) and higher (rat 2) than the dose later given to a human subject.

Breath CO_2 was collected continuously, in fractions, for the first 30 hr. After elapse of this time, samples were collected intermittently until the sixth day, when the animals were killed in order to isolate the blood haem. Very little radioactivity was found in the breath CO_2 after the first day. In rat 1, which had the lower dose, relatively less of the radioactivity was excreted in the urine than in the breath and faeces. In rat 2 this situation was reversed. Both animals excreted altogether about two-thirds of the radioactivity. The results of this experiment are set out in Table 1.

The radioactivity found in the haemin prepared from the blood of the rats is shown in Table 2. It may be seen that the small fraction of the administered ALA which is incorporated does not appear to vary with the dose. Additional data, previously obtained with necessarily very much

Table 1. *Excretion of radioactivity by rats which had received* [1:4-$^{14}C_2$]*ALA*

All samples were counted as $BaCO_3$ by method *C*, except the first three breath samples of rat 2, which were counted by method *B* (see Experimental section). Rat 1 received 0·0045 m-mole of ALA/100 g., and rat 2 0·043 m-mole of ALA/100 g. The total radioactivities administered were 0·268 and 2·41 μc respectively. A dash indicates that no urine or no faeces were produced in the period concerned.

	Time after dose (hr.)	Cumulative percentage of the total radioactivity excreted		
		Breath CO_2	Faeces	Urine
Rat 1	3	11·0	—	—
	6	13·2	0·2	21·1
	22·5	16·9	15·5	24·8
	29	17·0	—	—
	51	17·0	17·4	25·1
	70·5	17·0	17·9	25·2
	120	17·0	18·9	25·3
		Total ^{14}C excreted by rat 1 in 5 days = 61·2%		
Rat 2	3·5	4·6	—	—
	6·3	7·8	—	34·7
	24	10·9	10·3	37·3
	51	11·3	—	37·5
	72	11·5	14·5	37·6
	120	11·9	14·8	37·7
		Total ^{14}C excreted by rat 2 in 5 days = 64·4%		

Table 2. *Incorporation of isotopic ALA into haemoglobin haem*

Rats were killed on the sixth day after intraperitoneal injection of isotopic ALA. The data from which the incorporation of [^{15}N]ALA has been calculated have been taken from the previous paper (Berlin *et al.* 1956). For calculation of the percentage incorporation, the observed isotope contents of the haemin have been corrected for loss of $\frac{6}{16}$ of ^{14}C or $\frac{4}{8}$ of ^{15}N which must have occurred during the conversion of PBG into protoporphyrin.* It was calculated that the rats contained the equivalent of 30 mg. of haemin/100 g. (see text).

Rat (no.)	Compound injected	Dose (m-mole/100 g.)	Isotope content observed	Percentage of the ALA used for haem synthesis
1	[1:4-$^{14}C_2$]ALA	0·0045	$1·947 \times 10^{-3}$ μc/m-mole	0·11
2	[1:4-$^{14}C_2$]ALA	0·043	$1·956 \times 10^{-2}$ μc/m-mole	0·12
17 + 18	[^{15}N]ALA	0·20	0·018 atom % excess	0·11
30 + 31	[^{15}N]ALA	0·50	0·045 atom % excess	0·11

* 16 ^{14}C atoms or 8 ^{15}N atoms in 8 molecules of the isotopic ALA are required for producing one protohaem molecule; 6 carboxyl-^{14}C atoms or 4 ^{15}N atoms are eliminated in the process.

larger doses of [^{15}N]ALA, have been included. The total radioactivity incorporated into haem was calculated for a haem content equivalent to 30 mg. of haemin/100 g. This figure was calculated from the blood-haemoglobin concentration of 17·1 g./100 ml., determined by the method described, assuming a blood volume of 4·6 ml./100 g. (Berlin, Huff, Van Dyke & Hennessy, 1949).

Experiments with cockerels

It was considered possible that the different excretory system of birds, in particular the smaller volume of water lost, might cause an increased utilization of administered ALA for synthesis of haem by decreasing the amount lost in the urine compared with that lost in mammals. This was not found to be the case, since the amount of [1:4-$^{14}C_2$]-ALA incorporated into haem by a cockerel was approximately the same as in mammals, assuming, in the absence of accurate data on the blood volume of birds, that the haem content/100 g. was the same as that of rats (Table 3). An equimolar dose of [1:4-$^{14}C_2$]succinic acid, having a similar specific activity, was given to a second cockerel. In contrast to glycine (Neuberger & Scott, 1953), incorporation of the labelled succinate into haem was over 100 times less than that of correspondingly labelled ALA (Table 3).

Table 3. *Incorporation of ALA or succinate into haem by cockerels*

[1:4-$^{14}C_2$]Succinic acid and [1:4-$^{14}C_2$]ALA contained 32·0 and 29·5 μc/m-mole respectively. Both compounds were injected intraperitoneally (0·02 m-mole/100 g.) and the birds were killed after 6 days. It was assumed that the cockerels contained the same proportion of haem as rats (see text). The haemin radioactivity has been corrected for loss of ^{14}C (see Table 2), and was assayed by method *D*.

Bird (no.)	Compound injected	Corrected haemin radioactivity (μc/mole)	Percentage of the compound used for haem synthesis
19	[1:4-$^{14}C_2$]ALA	12·2	0·095
20	[1:4-$^{14}C_2$]succinic acid	0·115	0·00083

Administration of [1:4-$^{14}C_2$]*ALA to man*

A solution of [1:4-$^{14}C_2$]ALA (1·00 g. of the hydrochloride) was swallowed by one of us (J.J.S.) at 7.00 a.m., and no food was taken until midday, in order not to hinder the absorption. This dose was equivalent to 0·007 m-mole/100 g. Frequent samples of blood, breath, urine and faeces were taken throughout the day, and afterwards daily for 84 hr. No method for re-isolation of ALA from urine was available at this time, so that the specific activity of the excreted ALA could not be determined. Urinary PBG, faecal porphyrins and stercobilin were isolated and estimated by the methods described.

The amounts, specific radioactivities and isotopic dilutions of the several compounds excreted and isolated are shown in Table 4. It can be seen that dilution of the newly formed PBG and protoporphyrin by endogenous material was marked, though not great. The significance of the constancy of the activity of the protoporphyrin isolated on 4 successive days is discussed below. In addition to protoporphyrin, small amounts of coproporphyrin I (about 0·5 mg. of the methyl ester, m.p. 218–220°) were isolated from the daily faeces samples. The

Table 4. *Amounts and specific activities of compounds isolated*

[1:4-$^{14}C_2$]ALA (1·001 g.; 0·0075 m-mole/100 g., having 0·8111 μc/m-mole) was taken orally by a man weighing 79·4 kg. Total radioactivity received was 4·85 μc. The radioactivity of the haemin was significant but very low (0·0702 ± 0·03 disintegration/min./mg. of $BaCO_3$ by method *C* after combustion); for this reason the haemin activity could not be determined with the same precision as those of other compounds, which were assayed by method *A*. In the calculations required to obtain the figures in the last column, the observed activities (column 4) have been divided by the number of ALA residues in the compound concerned, the activities of tetrapyrrolic compounds having first been corrected for loss of ^{14}C (see Table 2).

Compound	Period over which sample was collected (hr. after dose)	Amount excreted (mg. of the hydrochloride)	Specific activity (μc/m-mole)	Observed specific activity as percentage of the maximum value
ALA	0–24	319	Not isolated	?
PBG	0–8	29·4	1·27	78·1
Protoporphyrin	0–12	4·9	2·39	59·0
	13–36	12·9	2·36	58·3
	37–60	3·2	2·38	58·7
	61–84	4·1	2·36	58·3
Stercobilin	0–12	188	0·0036	0·1
	13–36	212	0·1200	3·0
	37–60	74	0·1050	2·6
	61–84	277	0·0459	1·1
Haemin	After 5 days	—	$2·1 \times 10^{-4} \pm 43\%$	0·005

quantities available were insufficient for determination of the specific activity after purification. The rates of excretion of radioactivity in the various fractions, and in specific compounds, have been plotted in Figs. 2–4. The total radioactivity in any fraction (Table 5) is therefore equal to the integral after zero time of the corresponding curve in Figs. 2, 3 or 4. The percentage of the total radioactivity associated with each fraction is shown in Table 5. The haemoglobin concentration was found to be 17·0 g./100 ml. of blood, and the blood volume was taken to be 68·6 ml./kg. (Berlin, Lawrence & Gartland, 1950). The total haemoglobin haem was therefore equivalent to 355 mg. of haemin/kg., and the total blood volume was 5·45 l., since the subject weighed 79·4 kg. Although the urinary excretion of ALA roughly paralleled the total urine ^{14}C (Fig. 2), a substantial proportion of the urinary ^{14}C, particularly that part excreted after 6 hr., has not been accounted for as specific compounds (Table 5). Although additional contributions may be expected from urea ^{14}C and $H^{14}CO_3^-$ ion, in view of the rate of ^{14}C excretion by the lungs (Fig. 3), it is likely that other, more specific metabolites of ALA were present. Similar considerations apply to the faecal ^{14}C (Fig. 4 and Table 5), except that the total faecal ^{14}C in the final period was entirely due to protoporphyrin and stercobilin.

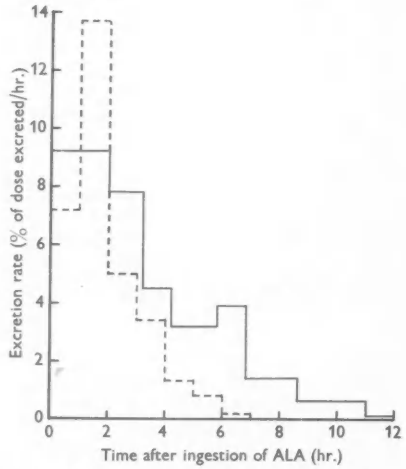

Fig. 2. Urinary-excretion rates of ALA (broken line) and of total ^{14}C (continuous line) by a human subject after ingestion of [1:4-$^{14}C_2$]ALA. The subject (J.J.S.) received 0·0075 m-mole of [1:4-$^{14}C_2$]ALA (29·5 μc/m-mole)/100 g. body weight. The data for ALA excretion were obtained subsequently with the same subject given an exactly similar dose of non-isotopic ALA. Total urinary ^{14}C was assayed by method *C*.

The appearance of ^{14}C in the blood was followed directly. Blood samples were taken at intervals, and the radioactivity of the packed red cells was measured separately from that of the plasma, in order to see whether ALA or a metabolite was actively taken up from the plasma by the red cells. The change in the activities in the red cells and in the plasma with time is shown in Fig. 5*a*, and the ratio of these activities is shown in Fig. 5*b*. At the peak of the red-cell curve in Fig. 5*a*, after 3·3 hr., both the whole blood and the red cells contained the maximum activity observed: 14·8 % of the total radioactivity administered was in the whole blood, and 11·2 % was in the red cells. It is apparent from Fig. 5*b* that an early component of the plasma ^{14}C is being actively taken up and partially retained by the red cells.

From the curve for plasma ^{14}C (Fig. 5*a*) the mean plasma ^{14}C concentrations during the periods of urine collection can be estimated. This permits calculation of mean plasma clearances of ^{14}C over the appropriate periods. Results of these calcula-

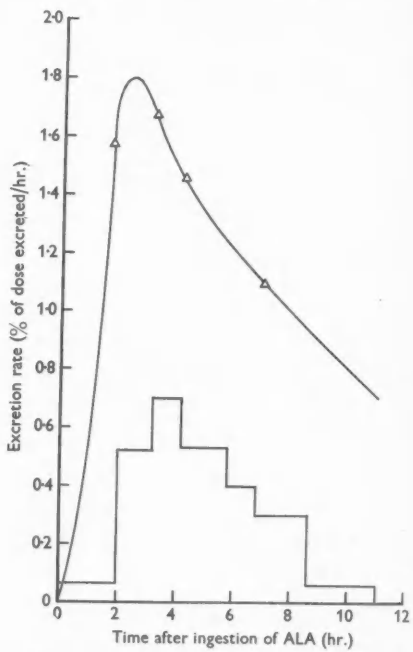

Fig. 3. Excretion rates of ^{14}C in the breath and in urinary PBG, after ingestion of [1:4-$^{14}C_2$]ALA by a human subject. Conditions as in Fig. 2. □, PBG ^{14}C (assayed by method *A*); △, breath $^{14}CO_2$ (assayed by method *C*). After 26 hr. the rate of excretion of ^{14}C in the breath was zero; this point is not shown in the figure.

tions are given in Table 6, where also the concentrations of total ^{14}C and of (ALA + PBG)-^{14}C in the urine are compared. It may be seen that the clearance values fall into three groups, corresponding approximately to the periods of urinary excretion of ^{14}C derivable as follows: (1) mainly from ALA + PBG; (2) from ALA + PBG + unknown compounds; (3) mainly from unknown compounds.

Administration of [^{15}N]ALA to man

In order to test whether a comparable dilution of newly formed PBG was obtained in a different subject, and at a different dose level, a further experiment was performed, with [^{15}N]ALA. The hydrochloride (3·00 g., containing 0·999 atom % of excess ^{15}N) was administered to a second male subject (K.D.G.), weighing 85·6 kg.; the severe erythema which resulted from this large dose of ALA has been described (Berlin *et al.* 1956). The proportion of ALA excreted in the urine was 52·3 %, and PBG containing 0·944 atom % of excess ^{15}N was isolated from the urine voided between 0 and 8 hr. (see Table 7).

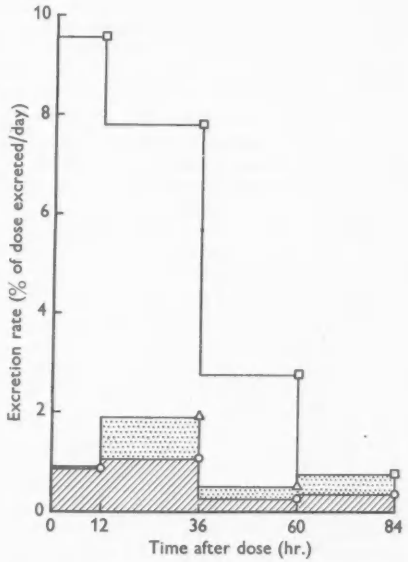

Fig. 4. Excretion rates of ^{14}C in the faeces, after ingestion of [1:4-$^{14}C_2$]ALA by a human subject. Conditions as in Fig. 2. □ (plain area), total faecal ^{14}C (assayed by method *C*); △ (stippled area), protoporphyrin ^{14}C; ○ (hatched area), stercobilin ^{14}C (both assayed by method *A*).

DISCUSSION

It is apparent from the results presented here and in the previous paper (Berlin *et al.* 1956) that the general pattern of the metabolism of administered aminolaevulic acid is very similar in the rat (Table 1) and in man (Table 5); this similarity extends to birds, at least as judged on the basis of the degree of incorporation of aminolaevulic acid into haem (Tables 2 and 3). Quantitatively, the main features of aminolaevulic acid metabolism, based on the results in man, are a rapid excretion of unchanged material (about 30 %) and a transformation into other compounds, some of which (about 50 %) are excreted rapidly, while others (about 20 %) are still in the body after 4 days.

Faecal radioactivity

In man, the faecal radioactivity is believed to arise entirely from metabolized rather than from unabsorbed aminolaevulic acid, since similar fractions of the dose were recovered from the faeces of the rats, to which the dose had been administered parenterally. The small fraction of the faecal radioactivity accounted for has already been mentioned: it is possible that, had the isolation of

Table 5. *Recovery of radioactivity after giving* [1:4-$^{14}C_2$]*ALA to man*

The conditions of the experiment from which the following data are compiled have been summarized in Table 4. It was calculated that the subject contained the equivalent of 355 mg. of haemin/kg. (see text). Since the measurements of breath $^{14}CO_2$ were discontinuous, the total radioactivity excreted by this route was obtained by measuring the area from 0 to 24 hr. below the interpolated curve in Fig. 3. The value for the maximum radioactivity that can have been excreted as ALA was calculated from the amount of this compound excreted, by assuming that there had been no dilution by endogenous ALA; the minimum value was obtained by assuming that the dilution of the PBG was entirely due to endogenous ALA.

Fraction	Percentage of total radioactivity: In the whole fraction after 84 hr.	Percentage of total radioactivity: In specific compounds isolated
Breath CO_2	14·8	—
Whole urine	48·6	—
ALA	—	24·5–31·9
PBG	—	3·4
Unaccounted for	—	13·3–20·7
Whole faeces	16·1	—
Protoporphyrin	—	2·1
Stercobilin	—	1·5
Unaccounted for	—	12·5
Blood haem	0·24	—
Total	79·7	

porphyrins and bile pigment been carried out after previous sterilization of the alimentary tract with antibiotics, there might have been a greater recovery of ^{14}C in the compounds isolated. Watson (1938) reported that individual stercobilin excretion by a group of adults varied between 40 and 280 mg./day, whereas the daily bilirubin production by a 70 kg. man should be about 250 mg.; it is obvious therefore that the stercobilin excreted may represent only a small fraction of the bilirubin produced.

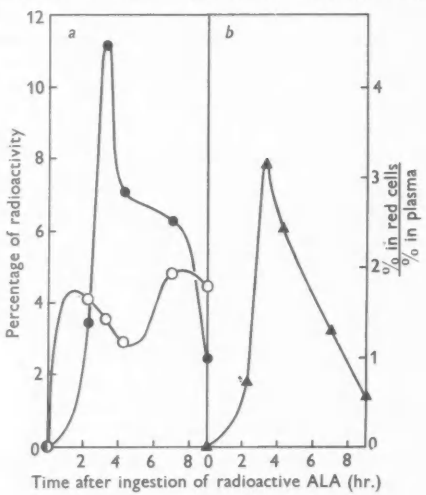

Fig. 5. (*a*) Variations of total red-cell and plasma ^{14}C with time after ingestion of [1:4-$^{14}C_2$]ALA by a human subject. Conditions as in Fig. 2. The haemotocrit values were 50 for all blood samples taken during the period shown. Total ^{14}C was assayed by method *C*. The blood volume was taken to be 5·45 l. (see text). ●, Red cells; ○, plasma. (*b*) Changes in the distribution of ^{14}C between red cells and plasma with time. Points on this curve are derived from the ratios of the values of ordinates in Fig. 5*a*.

The specific activity of the abnormally large quantities of protoporphyrin formed (totalling 25 mg.) remained approximately constant throughout the experiment. This suggests that all the protoporphyrin was formed at about the same time, and that an enterohepatic circulation caused the distribution of this material over the faeces produced during the subsequent 3 days. Enterohepatic circulation of naturally occurring porphyrins does not appear to have been described before, though Rothemund, McNary & Inman (1934) and Rothemund (1935) have detected phylloerythrin and phylloporphyrin in ox bile. The presence of naturally occurring porphyrins in human bile is well recognized, but evidence that they are reabsorbed has hitherto been lacking. In view of the constant radioactivity of the protoporphyrin found in the present study, either this compound, after reabsorption into the portal blood, must have failed to equilibrate with the protoporphyrin (if any) being synthesized normally by the liver before re-excretion into the bile took place, or else free protoporphyrin is not a normal intermediate in the hepatic formation of haem proteins such as catalase. This latter interpretation is preferred, since liver cells are known to be extremely permeable to most small molecules. That aminolaevulic acid gives rise to porphyrins *in vivo* has also been demonstrated without the use of isotopes by cannulation of the bile duct of a rat after parenteral injection of aminolaevulic acid (Scott, 1955). As in the present experiments, only traces of coproporphyrin, but large quantities of protoporphyrin, were formed. It is not possible to decide from the data given here whether the porphyrin excreted was formed in the liver or extrahepatically. In view of the photosensitivity of human subjects given ALA (Berlin *et al.* 1956) it seems likely that there were porphyrins present peripherally; recently it has been found that porphyrins are present in the skin of rats after injection of aminolaevulic acid intra-

Table 6. *Plasma clearance of radioactivity*

Values of plasma clearance of ^{14}C have been calculated for each period of urine collection during the human experiment with [1:4-$^{14}C_2$]ALA (for details see Table 4 and Fig. 2). The clearance is given by $U \times V/P$, where V is the rate of urine flow, and U, P are the concentrations of ^{14}C in urine and plasma respectively. The mean plasma concentrations for the appropriate period of urine collection have been interpolated from the measurements shown in Fig. 5*a*, assuming a plasma volume of 2·73 l. In calculation of the values shown in the fourth column, it has been supposed that the ALA-^{14}C was not diluted, and that PBG had the activity shown in Table 4; these suppositions are not entirely correct since the concentration of (ALA + PBG) ^{14}C is apparently greater than U in the first period.

Period (hr.)	V (ml./min.)	U (% of dose/ml.)	Urinary (ALA + PBG) ^{14}C (% of dose/ml.)	10^3P (% of dose/ml.)	^{14}C clearance (ml. plasma/min.)
0–2	1·22	0·125	0·144	1·43	106
2–3·17	1·36	0·096	0·068	1·41	93
3·17–4·17	2·48	0·045	0·033	1·21	92
4·17–5·83	1·05	0·051	0·018	1·34	40
5·83–6·83	7·56	0·009	0·002	1·69	40
6·83–8·58	1·31	0·018	0·003	1·72	14

peritoneally, particularly if the animals are irradiated (Jarrett, Rimington & Willoughby, 1956). It seems possible therefore that there was at least some extrahepatic synthesis of protoporphyrin from aminolaevulic acid, unless the porphyrin was incompletely excreted by the liver.

The rapid appearance of radioactivity in the stercobilin, considerably higher than in the circulating haem, is in agreement with previous findings that a small fraction of labelled glycine is converted rapidly into bile pigment by normal subjects (London, West, Shemin & Rittenberg, 1950; Gray *et al.* 1950). The prolonged excretion of radioactive stercobilin was to be expected from the known enterohepatic circulation of this compound (McMaster & Elman, 1925).

Origin of the radioactive carbon dioxide

In both rats and in the human subject a considerable amount (about 15 %) of the radioactivity from [1:4-$^{14}C_2$]aminolaevulic acid was lost in the breath. Two possible origins of this $^{14}CO_2$ will be considered. In the conversion of the side chains of porphobilinogen into those of protoporphyrin, $\frac{6}{16}$ of the labelled carbon atoms of the original aminolaevulic acid must be lost as CO_2, as are those of similarly labelled succinic acid (Shemin & Wittenberg, 1951). Were all the breath $^{14}CO_2$ to have arisen in this way, it would require over 40 % of the aminolaevulic acid to have been converted into protoporphyrin, haem or derived compounds such as stercobilin; and this figure does not allow for $^{14}CO_2$ so liberated but not excreted by the lungs. The sum of the radioactivity in the excreted tetrapyrroles and in haem was only 4 % of the radioactivity given (Table 5). However, the sum of this radioactivity and that unaccounted for in the urine and faeces lies between 33 and 40 %. If the radioactivity unaccounted for were due to the presence of protoporphyrin catabolites, it would indicate that the breath $^{14}CO_2$ had resulted from protoporphyrin synthesis. An alternative origin is from degradation of the aminolaevulic acid to α-oxoglutarate or succinate (cf. Shemin & Russell, 1953), when a rapid release of $^{14}CO_2$ would be expected from oxidation of these compounds in the tricarboxylic acid cycle. Consideration solely of the percentage of the dose involved cannot distinguish between these alternative processes until components of the non-specific fraction of faecal and urinary ^{14}C have been identified. A similar ambiguity was noted when the mode of origin of isotopic ammonia from [^{15}N]aminolaevulic acid was discussed (Berlin *et al.* 1956).

Consideration of the times of appearance of the several radioactive compounds is more indicative. It may be seen in Fig. 3 that the peak of ^{14}C liberation precedes that of excretion of radioactive porphobilinogen in the urine. The peak of $^{14}CO_2$ liberation in porphyrin synthesis was therefore still later; it has in fact been shown that the peak of excretion of porphobilinogen in the urine precedes the peak of protoporphyrin excretion in the bile, after aminolaevulic acid has been given to rats (Scott, 1955). It is therefore concluded that the larger part of the breath $^{14}CO_2$ arose from oxidative degradation; this would accordingly represent the extent to which the aminolaevulic acid took part in any series of reactions such as the cycle proposed by Shemin & Russell (1953). The total amount of administered aminolaevulic acid converted into protoporphyrin and derivatives should be proportional to the difference between the total pulmonary ^{14}C excretions after [1:4-$^{14}C_2$] and [2:3-$^{14}C_2$]aminolaevulic acid respectively have been given, since $^{14}CO_2$ should not be lost from the latter compound during conversion into protoporphyrin.

Significance of the changes in the radioactivity of the blood

The plasma ^{14}C rose rapidly during the first 2 hr. and did not change greatly for the remainder of the experimental period of 9 hr. (Fig. 5*a*). The urinary ^{14}C, on the other hand, decreased sharply and continuously after the first 2 hr. period. In order to compare radioactivities in plasma and urine respectively, plasma clearance values for ^{14}C were calculated (Table 6), and ^{14}C was thus treated as if it were present in one substance only. The ^{14}C clearance values during the first 4 hr. were close to the average inulin clearance value of 94 ml./min. for adult man (Walser, Davidson & Orloff, 1955). If the unlikely possibility is neglected that the first three observed figures are the fortuitous result of the presence in plasma of one substance which was actively secreted by the tubules while another substance was filtered and partially reabsorbed, it must be concluded that most of the radioactive material present in plasma during the first 4 hr. consisted of a substance or substances removed by glomerular filtration and not reabsorbed. Practically the whole of the radioactivity of the urine for the first 2 hr. period was due to aminolaevulic acid, whereas for the third and fourth hours this compound accounted for two-thirds and half respectively of the urinary radioactivity. It is therefore concluded that the changes in radioactivity in plasma and urine during the first 3 hr. represent largely the absorption of aminolaevulic acid from the gut and its excretion by the kidney through glomerular filtration, no appreciable reabsorption by the tubules taking place. However, during the third hour porphobilinogen was excreted in significant quantities; Goldberg & Rimington (1954) have shown that this substance is eliminated only by glomerular filtration and is not reabsorbed or excreted by the tubules. A small amount of the aminolaevulic acid (2–3 %,

Fig. 3) was degraded in the first 2–3 hr., yielding carbon dioxide which appeared in the breath. It is evident from the data of Stadie & O'Brien (1937) that approximately 8 % of the total blood CO_2 is removed every minute by the lungs; $^{14}CO_2$ produced will accordingly be lost from the blood rapidly. It may be calculated that not more than 10 % of the radioactivity of the plasma at 3 hr. can have been due to $^{14}CO_2$, assuming that the breath CO_2 collected at this time was representative of the CO_2 remaining in the blood. There may have been other radioactive substances present in the blood during the first 2–3 hr., but their concentration is likely to have been small compared with that of aminolaevulic acid.

The red-cell ^{14}C increased up to the end of the third hour, fell sharply in the fourth hour, rather slowly in the subsequent 3 hr. and then again rapidly (Fig. 5*a*). The shape of this curve, considered in conjunction with the changes in plasma ^{14}C and urinary excretion of aminolaevulic acid and porphobilinogen, suggests that aminolaevulic acid entered the red cells, but was there converted into other compounds (*Z*). The hypothetical *Z* is believed to have diffused out of the red cell in significant quantities after the third or fourth hour, then to contribute to the secondary rise in plasma ^{14}C (Fig. 5*a*), and also to the radioactivity of the urine in the later part of the experiment.

This later period (hours 4–11) was characterized by the following: (*a*) the urine ^{14}C was diminishing, but only a small part of it could be accounted for by aminolaevulic acid and porphobilinogen; (*b*) the plasma ^{14}C remained high and thus the clearance values were steadily diminishing; (*c*) the breath $^{14}CO_2$ was decreasing. It would seem that during this period the plasma ^{14}C was due to substances including *Z*, which are metabolites of aminolaevulic acid. These substances were excreted partly in the urine, by a mechanism involving partial reabsorption or incomplete filtration; they may also have been excreted by other routes such as through the liver into the bile.

Significance of the isotope dilutions

Dilution of an administered labelled compound, or of its transformation products, by mixing with endogenous material can arise in two ways. First, there will be dilution if there is a measurable instantaneous concentration of endogenous material; secondly, there will also be dilution if there is a rapid endogenous production and simultaneous transformation of the appropriate compounds without measurable quantities of any but the final product ever accumulating. During active metabolism, therefore, the magnitude of the dilution will be a function partially of the length of time the system is in contact with the administered material.

When the specific activities of the compounds isolated in the first human experiment are compared (Table 4), it is clear that some mixing with endogenous material has occurred. It is not possible to obtain absolute values for the total endogenous materials without knowing how much of the dose actually mixed with these materials before being excreted or, alternatively, how much of the dose was converted into any compound under consideration. It may, however, be concluded that 22 % of the porphobilinogen and over 40 % of the protoporphyrin isolated had an endogenous origin. The fact that these two figures are different cannot be taken to indicate that there was equilibration with a reservoir of the intermediates between porphobilinogen and protoporphyrin, since it is not known that the two compounds were formed within the same period. It is certain only that there was mixing of some fraction of the dose with endogenous aminolaevulic acid or porphobilinogen or both.

It is reasonable to suppose that the time during which the labelled aminolaevulic acid was being incorporated into haemoglobin haem was not greatly different from the time during which it was being excreted either as such or after conversion into porphobilinogen. This time was about 8 hr. (Figs. 2, 3), during which $\frac{1}{360}$ of the total haem was renewed, assuming a mean red-cell life of 120 days. From the known total radioactivity in the haemoglobin haem it may accordingly be calculated that, had this labelled haem been incorporated during 8 hr., the mean specific activity of the aminolaevulic acid used would have been only 2 % of the initial activity. It may be concluded that the labelled porphobilinogen, protoporphyrin and stercobilin isolated were not made in the haemopoietic tissues, where utilization of the administered material was evidently slight. The constancy of the proportion of the isotopic aminolaevulic acid incorporated into haemoglobin haem (Tables 2 and 3), in contrast to the increasing proportion of porphobilinogen excreted as the dose is increased (Berlin *et al.* 1956), suggests that there is a rate-limiting step associated with the utilization of aminolaevulic acid by the erythroblasts.

The data presented here (Table 3) and in the previous paper (Berlin *et al.* 1956; Table 1) show that glycine and succinic acid, the co-equal precursors of aminolaevulic acid, are incorporated into haem 7 times more and 100 times less respectively than an equivalent dose of aminolaevulic acid. The explanation of this must lie primarily in the different rates at which these compounds are metabolized and excreted after administration, but the possibility that erythroblasts are less permeable to aminolaevulic acid than to glycine cannot be excluded.

Table 7. *Standard Dilution Capacities of subjects given isotopic aminolaevulic acid*

For definition of S.D.C. and other symbols, see equations (1) and (3). J.J.S. received [1:4-$^{14}C_2$]ALA; K.D.G. received [^{15}N]ALA. With both subjects the urinary excretion of ALA was complete in 8 hr.

Subject	Q (m-mole/kg.)	C_Q	P within 8 hr.	C	Observed dilution capacity	S.D.C. (μmoles of ALA/kg./day)
J.J.S.	0·075	0·811 (μc/m-mole)	4·20	0·634 (μc/m-mole)	63	55
K.D.G.	0·211	0·999 (% excess)	6·48	0·944 (% excess)	36·7	50

In order to compare the dilutions in the excreted porphobilinogen isolated after administering isotopic aminolaevulic acid to human subjects, an arbitrary measure has been employed. This measure, termed the 'dilution capacity', is the rate of production of aminolaevulic acid which would be necessary to cause the mean dilution observed in the urinary porphobilinogen, if all the aminolaevulic acid given mixed with this endogenous material over a period of 8 hr., the period within which excretion of aminolaevulic acid and porphobilinogen is virtually complete. The dilution capacity is measured in μmoles of ALA/kg./day, and is defined by the following equation (cf. Arnstein & Neuberger, 1951):

$$\text{Dilution capacity} = \tfrac{24}{8} Q(C_Q/C - 1), \qquad (1)$$

where Q is the dose of aminolaevulic acid (m-moles/kg.), C_Q is the initial isotope content and C is the subsequent isotope content of the aminolaevulic acid used for synthesis of the porphobilinogen isolated from urine voided between 0 and 8 hr. As defined by equation (1), however, the dilution capacity in this and similar cases is not independent of the size of the dose where this is sufficiently large to increase the cellular concentration for a limited period, thereby increasing the degree of saturation of enzymes concerned in the transformations. The fraction of a dose of aminolaevulic acid transformed into porphobilinogen increases steadily with the dose (Berlin *et al.* 1956). Therefore, if the dose is increased, a larger fraction must mix with endogenous material at the sites of porphobilinogen production, causing a smaller value for the dilution capacity. In order to diminish this variability, a 'Standard Dilution Capacity' (S.D.C.) is defined (equations 2 and 3) as the value of the dilution capacity which would be expected if 4·8% of the dose was excreted in the form of porphobilinogen (this is the proportion usually excreted within 8 hr. when about 1 g. of aminolaevulic acid is given orally to a 70 kg. adult).

$$\text{S.D.C.} = \frac{\%\text{ of dose as urinary PBG in 8 hr.}}{4{\cdot}8} \times \text{dilution capacity}. \qquad (2)$$

Combining with equation (1), this may be rewritten

$$\text{S.D.C.} = 0{\cdot}625P.Q(C_Q/C - 1), \qquad (3)$$

where P is the observed percentage transformation into urinary porphobilinogen in 8 hr. The S.D.C. has only relative significance: it is only indirectly related to the true rate of extramedullary aminolaevulic acid production.

Results from experiments summarized above, in which isotopic aminolaevulic acid, differently labelled, was given at different dose levels to two male subjects, may now be compared (Table 7). In spite of the many possible variables for which no allowance can yet be made, the two S.D.C. values are found to agree quite closely. Further, any substantial departure from these values for the S.D.C. might be expected to provide evidence for and a measure of an abnormality in the rate of extramedullary aminolaevulic acid synthesis or of porphobilinogen utilization; such has indeed proved to be the case in acute porphyria (Neuberger, Scott & Gray, 1954) and in photosensitive porphyrias (Scott & Gray, to be published). The similarity of the S.D.C., as determined by use of [1:4-$^{14}C_2$]aminolaevulic acid, to that determined by the use of the ^{15}N-labelled compound indicates that it is unlikely that there is any extensive reversible deamination of aminolaevulic acid in normal man.

SUMMARY

1. [1:4-$^{14}C_2$]Aminolaevulic acid (^{14}C-ALA) was administered parenterally to rats and cockerels and orally to a human subject. A similar proportion of the ^{14}C given was incorporated into haemoglobin haem by all three groups, regardless of the size of the dose of ALA, whereas incorporation of [1:4-$^{14}C_2$]succinic acid by cockerels was over 100 times less.

2. Administration of ^{14}C-ALA was followed by rapid elimination of ^{14}C in breath, urine and faeces, one-fifth to one-third being retained after 3 days. General findings concerning the elimination and metabolism of ALA, based on previous results using [^{15}N]ALA, were confirmed and extended. ALA is believed to be eliminated in the urine by renal filtration only.

3. This and subsequent paragraphs relate to data obtained from human experiments. Approximately two-thirds of the urine ^{14}C, but only one-quarter of the faecal ^{14}C, was present in identified compounds.

4. Radioactive porphobilinogen (PBG), protoporphyrin, stercobilin and haem were isolated and the specific activities compared. It is concluded that, of these compounds, the first three, which all had a relatively high specific radioactivity, had been synthesized outside the haemopoietic system. All four had undergone dilution by endogenous material.

5. Evidence has been obtained for an enterohepatic circulation of protoporphyrin in man. Large quantities of this compound, having constant radioactivity, were isolated from the faeces voided during 4 days after ^{14}C-ALA had been given.

6. The peak of pulmonary ^{14}C excretion preceded by 1 hr. the peak of urinary excretion of radioactive PBG. The greater part of the breath $^{14}CO_2$ is therefore believed to have arisen from degradation of ^{14}C-ALA, the remainder having resulted from conversion of this compound into protoporphyrin and derivatives.

7. The radioactivities of the red cells and plasma were measured separately at intervals after ingestion of ^{14}C-ALA. The resulting data suggest that ALA was absorbed from the plasma by the red cells and there converted into other compounds, excreted in the urine more slowly than ALA or PBG. Factors influencing the blood ^{14}C levels are discussed.

8. A 'Standard Dilution Capacity' (S.D.C.) is defined, which permits comparison of the relative rates of extramedullary production of ALA in different individuals. Similar S.D.C. values were obtained with two normal human subjects given [^{15}N]- and [1:4-$^{14}C_2$]-ALA respectively.

We are grateful to Dr B. M. Tolbert, Radiation Laboratory, University of California, Berkeley, and to Mr R. C. Holloway at this Institute for gas-counting of $^{14}CO_2$, to Mr G. Dickinson at this Institute for measurement of ^{15}N, and to Professor C. H. Gray and Dr D. C. Nicholson, Department of Chemical Pathology, King's College Hospital, London, for isolation of faecal tetrapyrroles. We also wish to thank the subjects K.D.G. and B.A.A. for their co-operation, and Mr F. Grover, Mrs Betty Higginson, Mr C. Hill and Mr B. Wright for technical assistance.

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The Determination of δ-Aminolaevulic Acid

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δ-Aminolaevulic acid (ALA) is an intermediate in the biosynthesis of porphyrins (Shemin & Russell, 1953; Neuberger & Scott, 1953) and may be involved in other pathways of glycine metabolism (Shemin & Russell, 1953). In the study of these problems it became necessary to devise methods for the identification and measurement of small amounts of ALA in biological materials.

Two different methods have been investigated. The first, based on the Knorr reaction for the synthesis of pyrroles (Knorr, 1886), depends on the condensation of ALA with a suitable carbonyl compound to form a product, presumably an α-unsubstituted pyrrole, which gives a red colour when treated with Ehrlich's *p*-dimethylaminobenzaldehyde reagent. A report of the application of such a method to the determination of ALA appeared while this work was in progress (Granick & Vanden Schrieck, 1955). The second method is somewhat similar to the Jaffé reaction for the determination of creatinine with alkaline picrate (Jaffé, 1886). This reaction can also be used for identifying ALA on paper chromatograms.

EXPERIMENTAL

Preparation of compounds

δ-Aminolaevulic acid hydrochloride, δ-succinimidolaevulic acid, methyl δ-succinimidolaevulate, δ-succinamidolaevulic acid, methyl δ-chlorolaevulate and methyl δ-aminolaevulate, prepared according to Neuberger & Scott (1954), were kindly supplied by Dr J. J. Scott. Aminoacetone and 2:5-dimethylpyrazine, prepared by the method of Gabriel & Pinkus (1893), were also furnished by Dr Scott. The preparation of 6-amino-5-oxohexanoic acid hydrochloride is described by Gibson, Neuberger & Scott (1955). β-Oxoadipic acid was synthesized by the method of Eisner, Elvidge & Linstead (1950). δ-Diethylaminolaevulic acid was kindly supplied by Smith & Nephew Research Ltd., Ware, Herts. The preparation of the diethyl ester and one of the two monoethyl esters (ethyl α-amino-δ-carboxy-β-oxovalerate) of α-amino-β-oxoadipic acid, the *N*-benzoyloxycarbonyl derivative of methyl α-amino-δ-ethoxycarbonyl-β-oxovalerate, δ-oximinolaevulic acid, δ-acetamidolaevulic acid and δ-amino-γ-hydroxyvaleric acid is described by Neuberger, Scott & Shuster (1956).

Porphobilinogen was isolated from human urine by the method of Cookson & Rimington (1954) after oral administration of ALA (Berlin, Gray, Neuberger & Scott, 1954).

Determination by the Knorr reaction with ethyl acetoacetate

Since ALA is an amino ketone an attempt was first made to use the method of Elson & Morgan (1933) for the determination of glucosamine. This consists in heating for 15 min. at 100° with 1% acetylacetone in 0·25 M-Na_2CO_3. In the case of glucosamine the resulting product gives a red colour with Ehrlich's reagent. In preliminary tests under these conditions ALA gave a stable red colour, the intensity of which was proportional to concentration in the range of 0·5–5·0 μmole. The maximum absorption was at 553 mμ. The recovery of added ALA from trichloroacetic acid supernatants of an extract of acetone-dried rat-liver powder was about 90%.

The usual conditions employed in the Knorr reaction are heating with 75% acetic acid or with 40% KOH (Knorr & Lange, 1902). The condensation of ALA with acetylacetone was carried out under similar conditions and was found to proceed considerably faster in 5 N-NaOH than in either 0·25 M-$NaCO_3$ or 50% acetic acid. Moreover, the colour produced with Ehrlich's reagent after heating with 5 N-NaOH was greatly intensified by the addition of ethanol. In an effort to establish optimum conditions the effect of varying heating time, acetylacetone concentration and alkali concentration was determined, but, because of difficulties with high and variable blank values and the lack of proportionality at concentrations below 0·5 mM, this procedure was abandoned. Trial of several other carbonyl compounds showed ethyl acetoacetate to give far more satisfactory results, and the method finally adopted is based on condensation with this reagent.

Reagents

Ethyl acetoacetate. No special purification of the reagent-grade ester is required. A 10% aqueous solution is readily prepared by warming and this is stable for at least 2 days at room temperature.

10 N-NaOH. No precautions for the exclusion of CO_2 seem necessary.

Ehrlich's reagent. A 2% solution of reagent-grade *p*-dimethylaminobenzaldehyde in 5 N-HCl is stable for at least 3 weeks at room temperature.

ALA standard. An aqueous solution of recrystallized δ-aminolaevulic acid hydrochloride is stable for several weeks at room temperature.

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General procedure

1·0 ml. of 10 % ethyl acetoacetate is added to the sample, containing 0·1–0·5 μmole of ALA in a volume of 1·0 ml., followed by 0·5 ml. of 10 N-NaOH. The solution is mixed and heated in a boiling-water bath for 2 min., then cooled, and 0·5 ml. of water is added, followed by 1·0 ml. of 5 N-HCl to neutralize the NaOH. Then 1·0 ml. of Ehrlich's reagent is added, and the pink colour which appears on mixing is intensified by the addition of 5·0 ml. of 95 % ethanol. After mixing, the tubes are allowed to stand for 10 min. at room temperature; the colour is then read against a reagent blank in a colorimeter with a green filter (500–570 mμ.) or in a spectrophotometer at 550 mμ. An EEL portable colorimeter (Evans Electroselenium Ltd., Harlow, Essex) was used for most measurements. Standards of about 0·1 and 0·2 μmole of ALA should always be estimated concurrently with every set of experimental samples.

RESULTS

Absorption spectrum of the colour

Maximum absorption under the conditions of the standard assay occurs at 550 mμ., as compared with 552 mμ. for the Ehrlich colour with porphobilinogen (Cookson & Rimington, 1954), and 553 mμ. for the condensation product of ALA with acetylacetone. The molar extinction coefficient in terms of ALA at 550 mμ. is 11 500.

Effect of varying different factors

Ethyl acetoacetate concentration. The colour produced increases with acetoacetate concentration up to a level of about 3 %. Even at the highest concentrations tested there was little or no colour in the reagent blank, as contrasted with acetylacetone, which tends to give very high blank values at higher concentrations of the diketone. The precipitation of sodium ethyl acetoacetate is avoided by the use of a 10 % solution of acetoacetate instead of the undiluted ester.

NaOH *concentration.* Maximum colour development is obtained with NaOH concentrations of about 2·5 N. While the colour obtained initially with concentrations down to 0·1 N is almost as intense as with 2·5 N-NaOH, it is weakened rather than strengthened by addition of ethanol, so that the final intensity is much less.

Heating time. The rate at which chromogen forms during heating at 100° is illustrated in Fig. 1. In other experiments it was found that at the end of 2 min. the reaction is about 70 % complete. Since volume changes resulting from evaporation during such a short period may be neglected, this period was chosen as a convenient heating time. Provided that suitable standards are run with each batch of tubes, slight variations in the time of heating need not affect the accuracy of the procedure.

Alcohol concentration. Ethanol is used to intensify the Ehrlich colour in the acetylacetone determination of glucosamine (Schloss, 1951). At a concentration of 20–50 % (v/v), it has a similar effect in the acetoacetate determination of ALA, increasing the colour yield by about 50 %. When the condensation is carried out under acid, neutral or weakly alkaline conditions ethanol either dilutes the colour or actually causes it to fade.

Proportionality of colour intensity to ALA concentration

Colour formation by this method is directly proportional to the amount of ALA present over the range 0·05–0·5 μmole (Fig. 2). This allows the

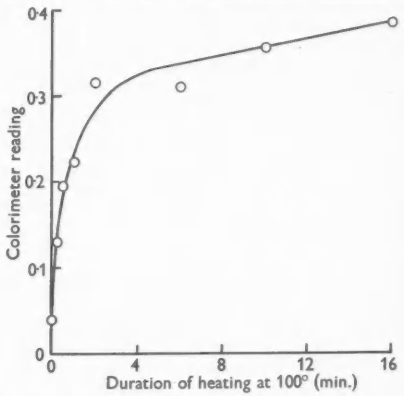

Fig. 1. Effect of heating time on colour yield by the acetoacetate method. Conditions were those of the standard assay (see text), with 0·23 μmole of ALA.

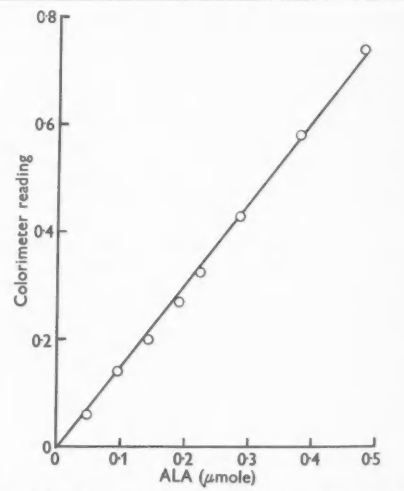

Fig. 2. Calibration curve for the ethyl acetoacetate method. Conditions were those of the standard assay.

Table 1. *Colour yields of some compounds compared with ALA*

Compound	Relative molar colour yield: Acetoacetate method	Relative molar colour yield: Picrate method
δ-Aminolaevulic acid hydrochloride	100	100
Aminoacetone hydrochloride	10	10
Diethyl α-amino-β-oxoadipate hydrochloride	6	31
α-Monoethyl ester of α-amino-β-oxoadipic acid hydrochloride	24	57
Ethyl methyl *N*-benzoyloxycarbonyl-α-amino-β-oxoadipate	0	0
Acetamidolaevulic acid	14	16
Methyl aminolaevulate hydrochloride	75	87
δ-Amino-γ-hydroxyvaleric acid lactone	0	0
δ-Oximinolaevulic acid	0	3
6-Amino-5-oxohexanoic acid hydrochloride	111	95
Methyl δ-chlorolaevulinate	0	8
δ-Diethylaminolaevulic acid hydrochloride	0	4
δ-Succinimidolaevulic acid	6	20
Methyl δ-succinimidolaevulate	6	23
δ-Succinamidolaevulic acid	138	35
Glucosamine hydrochloride	10	0
N-Acetylglucosamine	5	0
Acetone	0	1
Ethyl acetoacetate	0	2
α-Oxoglutaric acid	0	5
β-Oxoadipic acid	0	1
2:5-Dimethylpyrazine	0	0

determination of as little as 8 μg. of ALA-HCl. Sensitivity may be increased by reducing in proportion the volumes of the various reagents and measuring the colour in a spectrophotometer at 550 mμ.

Specificity and interfering substances

Other than compounds closely related to ALA, the only substances found to give rise to an appreciable colour in this determination were glucosamine and *N*-acetylglucosamine. The colour yield of both these substances is considerably less than that of ALA, but when they are known to be present it is advisable to use the picrate method described below. Table 1 gives, on a comparative molar basis, the colour yields of some compounds which have been tested. In addition it was found that pyruvic, glutamic and succinic acids, glucose and glycine gave negative results when tested at levels of 0·5–2·0 mg.

Porphobilinogen, which is formed enzymically from ALA in many tissues (Gibson *et al.* 1955), also gives a red colour with Ehrlich's reagent. Any colour due to porphobilinogen can be largely, but not completely, corrected for by running controls without added ethyl acetoacetate. Such a procedure greatly reduces interference by porphobilinogen in ALA determinations (Table 2). Extraneous interfering colours, such as caramel tints produced by heating solutions rich in carbohydrates, can also be corrected for by means of such controls.

Recovery from biological materials

The recovery of added ALA from human urine and from a haemolysate of chicken erythrocytes is essentially complete (Table 3). Similar experiments have shown that this method is suitable for the detection and assay of ALA in trichloroacetic acid supernatants of liver homogenates and other protein-containing extracts.

Table 2. *Determination of ALA in the presence of porphobilinogen by the acetoacetate method*

Solutions containing known amounts of ALA and porphobilinogen were analysed by the standard assay method, and the values thus obtained were corrected for colour due to porphobilinogen by subtracting the apparent values found in control determinations without ethyl acetoacetate. All values are expressed in micromoles.

Additions: ALA	Additions: Porphobilinogen	Apparent ALA: +Acetoacetate	Apparent ALA: −Acetoacetate	ALA (by difference)
0·095	0	0·095	0	0·095
0·190	0	0·190	0	0·190
0	1·77	0·078	0·074	0·004
0	4·43	0·160	0·145	0·015
0·095	4·43	0·202	0·145	0·057
0·190	1·77	0·238	0·067	0·171
0·190	4·43	0·308	0·140	0·168

Determination by the picrate method

In attempting to isolate the pyrrole formed by the Knorr reaction of ALA with ethyl acetoacetate it was observed that alkaline solutions containing ALA gave a deep-orange colour with picric acid. The Jaffé reaction for the determination of creatinine consists in the production of such a colour with alkaline picrate (Jaffé, 1886). The addition of concentrated HCl, which bleaches the picrate colour of creatinine, intensified and deepened the picrate

Table 3. *Recovery of ALA from biological materials by the acetoacetate method*

The urine was undiluted human urine. The blood filtrate was obtained by adding an equal volume of 10% trichloroacetic acid to a 10% aqueous solution of haemolysed chicken erythrocytes. A solution of ALA was added before deproteinization.

Sample	Vol. (ml.)	Calc. (μmole)	Found	Recovery (%)
Urine	0·2	0·190	0·194	102
Urine	0·2	0·095	0·103	108
10% trichloroacetic acid protein filtrate	1·0	0·189	0·186	98
5% trichloroacetic acid blood filtrate	0·4	0·151	0·132	88

colour of ALA. The colour with acetone, a source of interference in the Jaffé reaction, is also deepened by HCl, so that the test is not as specific as could be desired. However, the colours obtained with many carbonyl compounds, such as α-oxoglutarate, fade within 10–15 min. as contrasted with the colour from ALA, which is stable for at least several hours. Where interfering ketones are known to be present the ethyl acetoacetate test should be used instead of the picrate method.

The mechanism of colour formation in this test is still obscure. It is of interest that a similar reaction has recently been developed for the measurement of hydantoin-3-acetic acids (Selah & Berger, 1955).

Reagents

Picric acid. A saturated aqueous solution of reagent-grade picric acid was found to be most convenient. After exposure to light for several days picric acid solutions tend to give an orange colour with strong alkali (Owen, Iggo, Scandrett & Stewart, 1954). Since any such colour is completely bleached by the addition of HCl, no protection from light is necessary, and the solution can be kept for months at room temperature.

10 N-NaOH. No precautions for the exclusion of CO_2 seem necessary, although it is preferable to keep the solution clear.

General procedure

Saturated picric acid (0·1 ml.) is added to the sample containing 0·1–0·5 μmole of ALA in a volume of 1·0 ml. Because of its strong colour the picric acid solution should be pipetted accurately. The same results can be obtained with 0·3 ml. of 0·02 M picric acid. After mixing, 0·2 ml. of 10 N-NaOH is added and the solution is mixed again. Then 1·0 ml. of 5 N-HCl is added and, after mixing, the volume is brought to 5·0 ml. with water.

The colour develops almost immediately, and may be read in a colorimeter with a blue filter (450–500 mμ.) or in a spectrophotometer (450 mμ.) against a reagent blank. The absorption spectrum shows a rather broad peak in the region 440–500 mμ., with a molar extinction coefficient in terms of ALA of 6300 at 450 mμ.

Effects of varying different factors

Picric acid concentration. As in the Jaffé reaction, the colour developed is proportional to picric acid concentration up to a final concentration of 0·001 M (Bonsnes & Taussky, 1945). Above this level there is little change. The results obtained with more dilute solutions of picric acid (0·02 M and 0·002 M) were essentially similar.

Sodium hydroxide concentration. The colour increases sharply with NaOH concentration up to N-NaOH. Raising the concentration of alkali further has only a slight effect.

HCl *concentration.* The colour developed increases with increasing amounts of HCl up to the addition of about 3 m-moles. At higher concentrations there is a gradual decrease in colour strength. This is not due to fading, since the colour produced in concentrated acid seems to be as stable as that formed at lower HCl concentrations.

Effect of temperature. According to Hervey (1953), the colour intensity in the Jaffé reaction for creatinine varies appreciably with temperature at the time of reading. He recommended maintenance of a constant temperature during colour development and measurement. In the determination of ALA temperatures ranging from 15° to 40° were not found to affect the colour yield at time of measurement.

Specificity and intefering substances

The relative molar colour yield from a number of compounds which have been tested is given in Table 1. Glycine, glucose, and succinic acid in amounts of 10 mg. gave no appreciable colour under the conditions of the assay. Besides compounds related to ALA the only substances found to produce a stable colour were acetone and similar ketones. The picrate method cannot be used in the presence of amounts of acetone greater than 100 μg., and this feature is its chief limitation.

The picrate method can be used to measure ALA in the presence of porphobilinogen, unless the concentration of the latter greatly exceeds that of ALA (Table 4). Any interference resulting from the pink colour of most porphobilinogen solutions can be corrected for by running controls in which the HCl is added before the NaOH.

Proportionality of colour to ALA concentration

The plot of ALA concentration against colour as measured in the EEL colorimeter with a blue filter (440–500 mμ.) gives a straight-line relationship over a concentration range of 0·05–0·4 micromole (Fig. 3). Linearity in this concentration range is also obtainable in spectrophotometer readings at 450–470 mμ. In all cases it is desirable to run appropriate standards with each series of determinations.

Table 4. *Determination of ALA in the presence of porphobilinogen by the picrate method*

Solutions containing known amounts of porphobilinogen and ALA were analysed by the standard procedure; the results are expressed in terms of ALA.

Additions (μmoles)		ALA recovered
ALA	Porphobilinogen	(μmole)
0·095	0	0·095
0·190	0	0·190
0	1·77	0·006
0	4·43	0·030
0·095	4·43	0·132
0·190	1·77	0·190
0·190	4·43	0·202

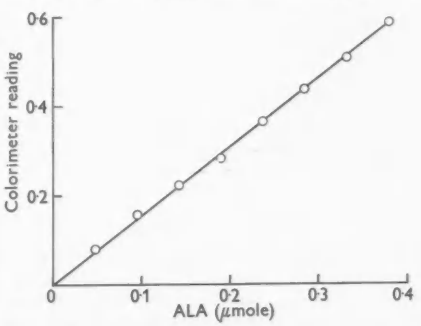

Fig. 3. Calibration curve for the picrate method. Conditions were those of the standard assay. Values are corrected for colour due to picric acid by running a control without added ALA.

Table 5. *Recovery of ALA from biological materials by the picrate method*

The samples were prepared as for Table 3

Sample	Vol. (ml.)	Calc. (μmole)	Found (μmole)	Recovered (%)
Urine	0·5	0·238	0·227	96
Urine	0·1	0·095	0·100	105
Urine	0·3	0·285	0·260	92
Urine	0·2	0·190	0·190	100
5% trichloroacetic acid blood filtrate	0·1	0·095	0·087	92

Application to paper chromatography

Although ALA gives a typical purple colour with ninhydrin in the assay of Moore & Stein (1948), it shows up in paper chromatograms developed with ninhydrin as a yellow spot (Berlin, Neuberger & Scott, 1956). ALA can also be detected on paper chromatograms by its pale-white fluorescence under ultraviolet light. As a further check, or in cases where these two methods are not readily applicable, ALA spots can be identified by the picrate method. The method consists of spraying with saturated picric acid, then with 5N-NaOH, and then with 5N-HCl, whereupon ALA and related compounds appear as reddish-orange spots which gradually fade to a light-brown colour. It is advisable to ensure adequate ventilation during spraying.

Recovery from biological materials

Because it can readily be applied to biological fluids the picrate test has been used extensively in this Laboratory in the study of ALA metabolism (Neuberger, Scott & Gray, 1954). Provided that appropriate blanks are used (a suitable procedure is to add the HCl before the NaOH), a reasonable amount of background colour should not interfere, and this test has been used to measure ALA in some darkly coloured urines from porphyric patients. The recovery of added ALA from human urine and from trichloroacetic acid filtrates of a blood haemolysate is shown in Table 5. As in the Jaffé reaction (Owen *et al.* 1954), it is advisable to neutralize acid filtrates before applying the picrate test.

DISCUSSION

The methods here described have proved adequate for studying the metabolism of ALA, and its excretion in certain cases of porphyria (Neuberger, Scott & Gray, unpublished observations). In identifying ALA it is desirable to confirm the results of these tests by paper chromatography and by means of the specific enzyme ALA dehydrase which condenses ALA to porphobilinogen (Gibson *et al.* 1955). In the absence of interfering substances the enzymic test can detect amounts of ALA as low as 0·01 μmole (about 1 μg.), and this estimation can be made semiquantitative by using a dilution which falls in the straight-line portion of the enzyme substrate saturation curve, i.e. between 10^{-5} and 10^{-4} M.

These methods also establish ALA and related compounds as possible interfering substances in the Elson & Morgan (1933) assay for glucosamine and the Jaffé (1886) reaction for creatinine.

SUMMARY

1. Two colorimetric methods are described for the determination of δ-aminolaevulic acid. Both are sensitive to 0·05 μmole.

2. One method is based on condensation with ethyl acetoacetate in 2N-NaOH to form a product which gives a red colour with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde).

3. The other method is a modification of the Jaffé picrate reaction and depends on the reaction of an alkaline picrate complex of ALA with HCl to give an orange-red colour.

4. The effects of varying different factors in the determination, specificity and application to biological materials are described for both methods.

I wish to thank Professor A. Neuberger, F.R.S., for his valuable advice and interest in this work, and for the hospitality of his laboratory; Dr J. J. Scott for providing many of the compounds tested in these assays; Mr B. L. Wright, Mr F. Grover and Mr C. Hill for technical assistance.

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The Penetration of Ornithine and Citrulline into Liver Slices

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The observations of Gornall & Hunter (1943) show that, at concentrations below 100 mg./100 ml., ornithine is uniformly a more effective catalyst of urea synthesis from ammonia in rat-liver slices than is citrulline. This difference in effectiveness appears to be at variance with what is to be expected of the cycle proposed by Krebs & Henseleit (1932), in which ornithine is transformed through citrulline into arginine, which, in turn, is hydrolysed to yield urea and more ornithine.

The increase in the steady-state rate of urea formation would be expected to be the same following equimolar additions of either ornithine or citrulline to systems catalysing this cycle, and during the approach to steady-state conditions the rate of increase in velocity of urea formation would be expected to be greater when citrulline is added than when ornithine is added. Therefore, the mean rate of urea formation over any period following the addition of a given amount of citrulline would be expected to be not less than the rate over the same period following the addition of an equimolar amount of ornithine.

It has been suggested by Krebs (1952) that the discrepancy between observation and this expectation is due to differences in accessibility of liver cells to the two amino acids. This suggestion appeared to receive some support from the observation of Christensen, Riggs, Fischer & Palatine (1952) that $\alpha\gamma$-diaminobutyric acid, the next lower homologue of ornithine, is powerfully concentrated by mouse liver.

The present investigation was undertaken to determine directly whether the greater effectiveness of ornithine could be accounted for in terms of differences of ease of penetration of ornithine and citrulline into liver cells.

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METHODS

Materials

Male and female rats of about 250 g. body wt. were used. All animals were fed on stock diets until required.

Liver slices were cut with the apparatus of McIlwain & Buddle (1953) at a nominal thickness of 0·225 mm. Immediately after cutting, the slices were washed with about 250 ml. of warm oxygenated Krebs bicarbonate–lactate medium (Krebs & Henseleit, 1932), the cutting and washing being complete within 10 min.

L-Ornithine hydrochloride and L-citrulline were obtained from Roche Products Ltd.

Incubation procedure

Approx. 100 mg. of slices was incubated at 37° in 10 ml. of the bicarbonate–lactate medium. The slices were kept suspended by a stream of gas mixture ($O_2 + CO_2$; 95:5) introduced at the bottom of the tube. The incubation tubes were fitted with air condensers. A trace of tributyl citrate was used to prevent frothing. Control experiments showed that this had no effect on the ability of liver slices to synthesize urea.

Fifteen minutes after adding ornithine or citrulline the slices were removed from the medium, blotted briefly on Whatman no. 54 filter paper, and placed in weighed bottles containing 1 ml. of 50% (w/v) trichloroacetic acid (TCA). A 5 ml. sample of medium was also pipetted into 0·65 ml. of 50% TCA. The slices were weighed and were then quantitatively transferred to a glass mortar with water, and ground up finely. The resulting suspension was quantitatively transferred to a weighed centrifuge tube with more water, and the solid separated by centrifuging. The whole of the supernatant (7–10 ml.) was taken for analysis. The dry weight of the slices was determined by reweighing the centrifuge tubes after they had been dried at 105° overnight. The trichloroacetic acid sublimes during the heating at 105°. Liver water was taken to be equal to the difference between fresh weight and dry weight of the slices.

Analytical methods

Direct colorimetric estimations. Ornithine was estimated according to the method of Chinard (1952) applied directly to TCA extracts, with a Spekker absorptiometer (Ilford spectrum filter no. 603). Citrulline was estimated by the method of Fearon (1939), as modified by Gornall & Hunter (1941), with a Pulfrich step photometer modified in a manner similar to that described by Stämpfli (1945).

Separation of ornithine and citrulline from interfering substances. Columns (5 cm. × 0·5 cm.) of Dowex 50 resin (2% cross-linkage, 200–400 mesh) were washed with 10 ml. of N-HCl followed by 10 ml. of water. The TCA extract to be analysed was transferred quantitatively to the column and allowed to run through, followed by 10 ml. of water. The column was washed with 5 ml. of 0·1 N-NH_3 soln. This did not displace ornithine or citrulline from a column of these dimensions, and it was thought that it might displace weakly held contaminant cations. The column was then washed with 10 ml. of water. All eluates to this point were discarded. The column was then washed with 10 ml. of N-NH_3 soln. followed by 10 ml. of water, and the eluates were collected. Tests showed that up to 1 mg. of ornithine or citrulline was quantitatively recovered from the column in these eluates. Finally, the eluates were evaporated to dryness at low pressure and temperature (not over 40°), and the residue was taken up in 0·5 ml. of water.

Paper electrophoresis. The apparatus used was similar to that described by Markham & Smith (1952). The addition of coils for the circulation of tap water through the CCl_4 bath in which the paper was immersed kept the temperature of the bath constant and made prolonged electrophoresis more satisfactory.

Rat liver contains a major unidentified ninhydrin-reacting component which runs close to citrulline at most pH values and is most readily separated from it at pH 3·2. Since ornithine is easily separated from other amino acids at this pH we have used electrophoresis for 3 hr. at 20 v/cm. in 0·045 M phthalate buffer of pH 3·2 as a routine procedure. Whatman no. 3 paper (18 cm. × 50 cm.) was used and 100 μl. spots of concentrated column eluate were applied. Table 1 gives the rates of migration of a number of amino acids in these conditions.

Colour development and measurement on paper. The method used was essentially that of Harris, Mittwoch, Robson & Warren (1954). We are grateful to Professor Warren for supplying the details before publication. The paper was dipped in 0·4% ninhydrin in 95% aqueous acetone (v/v) and dried. The colour was developed by heating at 60° for 30 min. This was most satisfactorily accomplished by wrapping the paper round a 10 l. bottle filled with water thermostatically maintained at 60°. After heating, the paper was dipped into a solution made by diluting 2 ml. of saturated aqueous $Cu(NO_3)_2$ and 0·4 ml. of 10% (v/v) HNO_3 to 100 ml. with acetone, and allowed to dry. The colours are intensified and made orange-red by this treatment. The coloured spots were cut out and the colour was eluted with methanol (5 ml. or more according to the intensity of the colour). The extinctions of the orange-red methanol solutions were measured at 510 mμ. in a Unicam spectrophotometer.

Calibration curves were prepared for solutions of the amino acids in the Krebs lactate medium and carried through the whole procedure, including addition of TCA, the resin stage, concentration, and separation by electrophoresis. Linear relations were obtained for both amino acids up to concentrations of 100 mg./100 ml. in the original solution. The same linear relations between concentration and extinction were found for ornithine and citrulline whether they were placed directly on paper or taken through

Table 1. *Migration of amino acids during electrophoresis*

Electrophoresis was carried out on a 50 cm. strip of Whatman no. 3 paper at pH 3·2, with a potential gradient of 20 v/cm. The values represent migration towards the cathode, uncorrected for endosmotic flow.

Aminoacid	Migration (cm.)
Aspartic acid	5·4
Glutamic acid	7·1
Citrulline	11·4
Glycine	16·5
Histidine	27·9
Arginine	30·0
Lysine	30·9
Ornithine	33·6

the above complex procedure. However, since the slope of the lines varied somewhat with different ninhydrin solutions, we always included standard solutions of ornithine and citrulline in every set of estimations to correct for this variable.

RESULTS

Measurement of the penetration of ornithine into liver slices

Chinard's method. Replicate sets of liver slices were incubated for 15 min. with 0, 11·8, and 23·6 μmoles of ornithine in the 10 ml. of medium, and ornithine concentrations were measured in the slices and medium by Chinard's (1952) method. Concentrations in the medium water and liver water (wet wt. − dry wt.) are compared in Fig. 1.

The linearity of the relation between the concentrations in liver water and in medium water suggests that ornithine enters by simple diffusion, but the large apparent ornithine content of the liver in the absence of ornithine in the medium, together with the known lack of specificity of the method (see Chinard, 1952), makes the interpretation of the data uncertain.

Paper electrophoresis. This more specific method was used in experiments in which the incubation period was again 15 min. and the initial ornithine concentrations were 0, 5·9, 11·8 and 17·7 μmoles in the 10 ml. of medium. Fig. 2 shows the results. These experiments give regular results, pointing unequivocally to a linear relation between concentrations in liver water and in medium water, and the apparent ornithine in the absence of added ornithine is low.

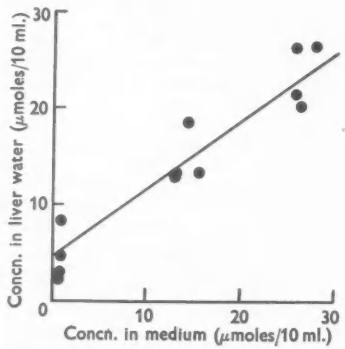

Fig. 1. Relation between liver and medium water concentrations of ornithine after incubation for 15 min., ornithine being estimated by Chinard's method. Slope $= 0{\cdot}704 \pm 0{\cdot}116$; intercept $= 4{\cdot}84$.

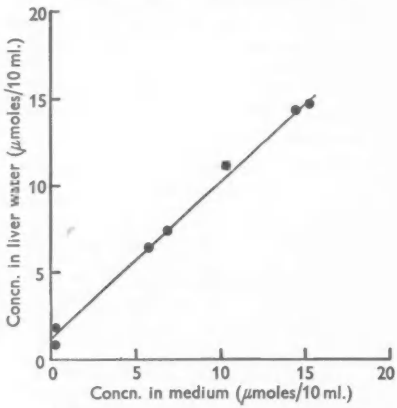

Fig. 2. Relation between liver and medium water concentrations of ornithine, paper electrophoresis being used to estimated ornithine. Slope $= 0{\cdot}928 \pm 0{\cdot}028$; intercept $= 1{\cdot}33$.

Measurement of the penetration of citrulline into liver slices

Fearon's method. The same general procedure of incubation with amino acid for 15 min. as was used in the ornithine experiments was followed in all the experiments with citrulline. Initial citrulline concentrations of 0, 11·4 and 22·8 μmoles/10 ml. were used. Fig. 3 gives the results of five experiments in which citrulline was estimated by Fearon's (1939) method. They show a linear relation between concentrations in medium and liver water, but the apparent citrulline in the liver water in the absence of added citrulline is rather high.

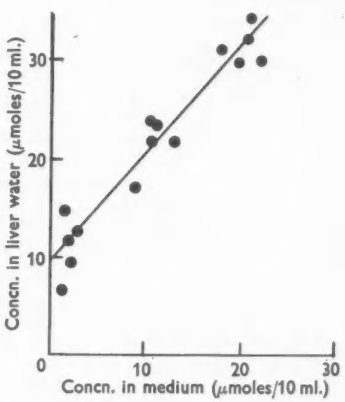

Fig. 3. Relation between liver and medium water concentrations of citrulline after incubation for 15 min., Fearon's method being used to estimate citrulline. Slope $= 1{\cdot}146 \pm 0{\cdot}092$; intercept $= 9{\cdot}31$.

Paper electrophoresis. The results are given in Fig. 4. There is no doubt that they are best described by a linear relation, and that the apparent citrulline in the absence of added citrulline is much lower than that found with Fearon's method of estimation.

Simultaneous penetration of ornithine and citrulline

We have shown (Bronk & Fisher, 1955) that ornithine and citrulline compete with one another in their catalytic effects on urea synthesis. Although the data for penetration are consistent with the hypothesis that both amino acids enter liver cells rapidly by simple diffusion, it was thought advisable, in view of the competition in catalysis of urea synthesis, to make sure that they did not compete for a pathway of entry into liver cells. Experiments were therefore made in which liver slices were incubated for 15 min. in medium containing both ornithine and citrulline. The results are presented in Table 2, in which the ratio of concentration in liver water to concentration in medium water is given for these experiments and for the earlier ones in which the amino acids were incubated singly with liver at the same concentrations. It is clear that the presence of the second amino acid does not affect penetration.

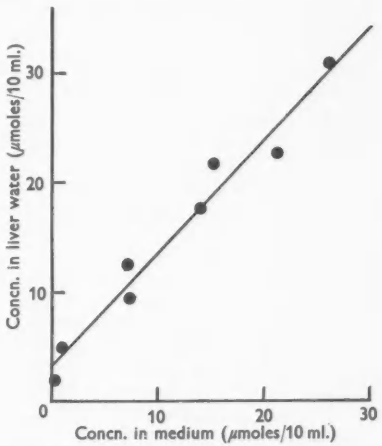

Fig. 4. Relation between liver and medium water concentrations of citrulline after incubation for 15 min., paper electrophoresis being used to estimate citrulline. Slope $= 1{\cdot}051 \pm 0{\cdot}087$; intercept $= 3{\cdot}01$.

Table 2. *Absence of competition between ornithine and citrulline for access to liver water*

All analyses were made by paper electrophoresis. Experiments with both amino acids were made with 5·9 μmoles of ornithine and 22·8 μmoles of citrulline added to 10 ml. of medium. Experiments with one amino acid added constitute all data from earlier experiments in which the final concentrations in liver water were comparable with those found in the experiments with two amino acids.

Additions to medium	Concn. in liver water/concn. in medium water: Ornithine	Citrulline
One amino acid		1·18
	1·01	1·05
	0·86	1·13
		0·99
Both amino acids	1·45	1·06
	1·05	1·01

Effect of time of incubation on penetration

Comparison of the results obtained with the direct colorimetric methods for the two amino acids with those obtained from paper-electrophoretic separation makes it clear that a large part of the apparent ornithine and citrulline found by the direct colorimetric methods in liver slices incubated in the absence of added amino acid must be some other contaminant material. The estimate of citrulline obtained by Fearon's method in these circumstances is three times that obtained by electrophoretic separation. The estimate of ornithine obtained by Chinard's method is between four and five times that obtained by the electrophoretic method.

However, in liver incubated in the absence of added amino acids the paper-electrophoretic technique still indicates appreciable amounts of apparent ornithine and citrulline, and the apparent concentrations of these two amino acids in liver water are much higher than their concentrations in the medium. This raises the question of whether there may be processes by which ornithine and citrulline can be concentrated in the liver cells. If such processes were occurring it would be expected that, at all concentrations of these amino acids in the medium, their concentration in liver water would rise in time above that in the medium. We have therefore made experiments identical with the 15 min. experiments (Table 2), except that the incubations were continued for 30 min. The data are given in Table 3. It is seen that the internal concentration of amino acid is not increased significantly in any instance above that to be expected in comparable 15 min. experiments. The failure of either amino acid to undergo further apparent accumulation in the liver in the experiments in which amino acid was added cannot be ascribed to lack of material in the medium: the ratio of volume of medium water to liver water in the experiments is about 20:1, so that enough apparent ornithine and citrulline were present to permit large increases in concentration ratio.

Recovery experiments

In order to interpret the penetration data it was necessary to know the completeness of recovery of the amino acids from liver slices. Recovery experiments were made by adding liver slices, incubated in the absence of added amino acid, to TCA in the usual fashion, and then adding a known amount of amino acid and carrying out the appropriate method of estimation. The results of these experiments are given in Table 4.

DISCUSSION

The uniformity with which the results of the penetration experiments can be described in terms of a linear relation means that one can say that every increment of concentration in the medium water produces a proportionate increment of the concentration in liver water. If it were possible to establish that all the apparent ornithine or citrulline in liver water in the absence of added amino acid was not ornithine or citrulline but a contaminant, then one could say that the concentration of the amino acid in liver water was directly proportional to that in the medium, the constant of proportionality being the slope of the regression line. This proviso is not fulfilled. But it is clear, from comparison of the estimates obtained by the Chinard and Fearon methods with those obtained by paper electrophoresis, that most of the apparent ornithine and citrulline found by the direct colorimetric procedures when no amino acid has been added to the medium must be contaminant material. Also (see Table 3) prolonging the period of incubation of the slices from 15 to 30 min. does not increase the concentration of apparent ornithine or citrulline in the liver water. Thus it is difficult to account for the apparent ornithine and citrulline on any hypothesis of active accumulation, and it seems most probable that the apparent residual material is a contaminant. The data of Table 3 are most simply explained on the assumption that the method of paper electrophoresis, though far more specific than the Chinard and Fearon methods, is still not perfectly specific for ornithine and citrulline, and that little or none of the amino acid estimated in the liver slices incubated in the absence of added ornithine or citrulline is in the form of ornithine or citrulline.

This is not a very important question in relation to the main purpose of this work since, even if active accumulation of the amino acids can occur to some extent, the data of Table 3 indicate that it must be occurring maximally at very low internal amino acid concentration. At the concentrations of ornithine and citrulline which are usually used in the study of urea synthesis the liver cells behave almost exactly as if the sole process governing entry was passive diffusion of the amino acids.

The slopes of the regression lines in Figs. 2–4 should therefore represent ratios of increments in amino acid concentration in liver water to increments in amino acid concentration in medium water, provided that there is nothing in liver which interferes with the estimation of added amino acid. Table 4 compares the regression coefficients (multiplied by 100 so that they give concentration in liver water as percentage of that in the medium) with percentage recoveries. It appears that liver contains something which interferes with Chinard's

Table 3. *Effect of incubation time on the penetration of ornithine and citrulline*

Amino acid	Amount added initially (μmoles)	No. of experiments	Concn. after 30 min. incubation (μmoles/10 ml.)		Concn. expected in liver water* (μmoles/10 ml.)
			Medium water	Liver water	
Citrulline	0	6	0·72±0·051	4·55±0·59	3·77±1·73
	22·8	4	20·50±0·52	19·14±0·74	24·55±1·16
Ornithine	0	6	0·10±0·017	1·36±0·06	1·42±0·24
	5·9	6	4·29±0·055	5·19±0·46	5·31±0·17

* These figures are calculated from the regression equations of Figs. 2 and 4 on the assumption that no further amino acid has entered after 15 min.

Table 4. *Comparison of the coefficients of regression of concentrations in liver water on concentrations in medium with the recoveries of ornithine and citrulline from rat-liver slices*

The numbers in parentheses denote the numbers of observations.

Amino acid	Analytical method	Regression coefficient (×100)	Percentage recovery
Ornithine	Chinard (1952)	70·4±11·7 (12)	74·5±7·6 (4)
	Paper electrophoresis	92·8±2·8 (7)	96·9 (2)
Citrulline	Fearon (1939)	114·6±9·3 (15)	94·5±3·7 (5)
	Paper electrophoresis	105·1±8·7 (8)	96·8 (2)

method for the estimation of ornithine. There is practically quantitative recovery of added ornithine by the paper-electrophoretic method, so that the low recovery by Chinard's method cannot be ascribed to binding of amino acid by liver solids.

Comparison between the regression coefficients and the percentage recoveries shows that in all instances the two agree within the limits of experimental error. Little or no weight can be placed on the differences which are shown, but it is of interest that the trend is for regression coefficients to be less than recoveries for ornithine and greater than recoveries for citrulline, so that there is a slight suggestion that citrulline penetrates into liver water more rapidly than ornithine.

We may conclude from this work that, at the concentrations of ornithine and citrulline ordinarily used in the study of urea synthesis, the intracellular concentration of both amino acids becomes indistinguishable from the external concentration within 15 min. of addition of the amino acid. In experiments such as those of Gornall & Hunter (1943) involving incubations of 2 hr. there can be no question of differences of cell permeability contributing appreciably to the differences in response to ornithine and citrulline.

It might, however, appear to be possible to invoke a permeability factor in connexion with penetration of the amino acids into intracellular particles, but such an explanation of the lower effectiveness of citrulline has to contend with the demonstration by Cohen & Hayano (1948) that the enzymes converting citrulline into arginine occur in the non-particulate fraction of the cytoplasm. That is, any intracellular permeability barrier would be expected to operate to the disadvantage of ornithine.

SUMMARY

1. Methods are described for concentration and separation by paper electrophoresis of ornithine and citrulline, and for their subsequent determination by the ninhydrin procedure of Harris *et al.* (1954).

2. These methods, and those of Chinard (1952) and Fearon (1939), have been applied to the study of the penetration of ornithine and citrulline into rat-liver slices.

3. Citrulline appears to penetrate a little more readily than ornithine. Both amino acids have almost the same concentration in liver water as in medium water after incubation for 15 min. at 37°.

4. No significant changes in the concentrations of the amino acids in liver water occur when the period of incubation is prolonged to 30 min.

5. The data do not support the view that ornithine is a more effective catalyst of urea synthesis than citrulline because it has easier access to liver cells.

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The Role of Ornithine and Citrulline in Urea Synthesis

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We have shown (Bronk & Fisher, 1956) that the anomalously greater effectiveness of ornithine, compared with that of citrulline, in catalysing synthesis of urea in rat-liver slices is not due to greater ease of access of ornithine to liver cells, as suggested by Krebs (1952). A possible alternative explanation is that ornithine and citrulline catalyse separate processes producing urea. This paper describes experiments made to test this possibility.

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METHODS

Biological material. Liver slices from rats and guinea pigs (average weights were 250 and 600 g. respectively) were used. The animals were fed on laboratory stock diet until

required. Liver slices were cut at a nominal thickness of 0·225 mm. on a McIlwain & Buddle (1953) tissue slicer, and were rapidly washed with about 250 ml. of warm, oxygenated Krebs's bicarbonate–lactate medium (Krebs & Henseleit, 1932) before use.

Incubation procedure. The liver slices were incubated in Krebs's bicarbonate–lactate medium containing NH_3 (100 μmoles/10 ml.) which was equilibrated with O_2+CO_2; (95:5). Vessels of the type shown in Fig. 1 were used for the incubation. Each vessel normally contained 10 ml. of medium and 100 mg. of slices, which were kept suspended by a gentle stream of gas from the capillary at the bottom of the tube. A trace of tributyl citrate prevented frothing, and did not interfere with urea synthesis.

At the end of incubation (in a water bath at 38°) the gas flow was stopped and a ground-glass stopper inserted into the mouth of the tube in place of the air condenser. The tube could then be disconnected from the gas line without loss of medium. The capillary was inserted into a half-bored rubber bung and the glass stopper removed. The tubes, which were designed to fit a standard 25 ml. centrifuge cup, were then centrifuged. The supernatant fluid was poured off, and the tube, which had previously been weighed, was re-weighed after thorough draining to give the wet weight of slices. The tube was dried in an oven at 105° overnight and reweighed to give the dry weight of slices.

The technique used here for obtaining liver weights probably gives higher estimates of weight, and therefore lower estimates of metabolic activity, than do more usual methods of recovering slices. We have washed slices which had been incubated for 60 min. in such a way that anything which can pass a 0·2 mm. mesh is carried off in the washings. About 10% of the solids can be separated from these washings by mild centrifuging. This fraction of the solids is included in the liver weight which we measure, but would not be included in an estimate of liver weight made by picking slices out of the medium.

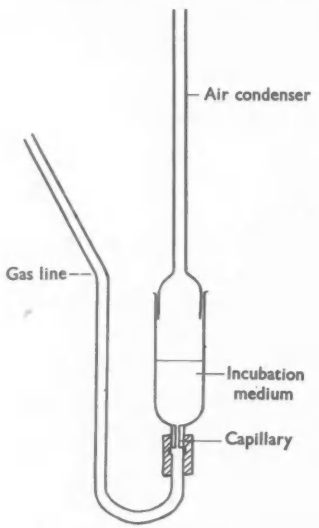

Fig. 1. Apparatus used for incubation and quantitative recovery of liver slices.

Estimation of urea. Urea was estimated by the method of Conway (1947) on 0·5 ml. samples of medium diluted with an equal volume of 0·2M phosphate buffer, pH 6·8. A 1:10 dilution of a glycerol extract of melon-seed meal ('Urease active meal from melon seeds', British Drug Houses, Ltd.) made by the method of Schmidt (1928) was used.

Ornithine and citrulline. L-Ornithine hydrochloride and L-citrulline (Roche Products, Ltd.) were used. These amino acids were estimated by the method of paper electrophoresis described by Bronk & Fisher (1956).

Units. Rates of urea synthesis are expressed in this paper as μmoles of urea formed/100 mg. dry wt. of tissue/hr. This unit is denoted by R_{urea}.

Reliability of the estimates of rates of urea synthesis. Since the method adopted for urea estimation depends on three independent measurements (free NH_3, total NH_3 after hydrolysis by urease, and NH_3 in the urease preparation) it was thought advisable to check the reliability of the analytical procedure by an analysis of variance of some of the observations. Three sets of data have been analysed. These consist of 9, 6 and 6 pairs of duplicate determinations in three different sets of conditions, three pairs of observations having been made on the same animal in each set. The three sets of data yielded mean R_{urea} values of 21·24, 18·95 and 2·53 respectively, with standard errors of means of duplicates of 1·69, 1·78 and 0·36. The standard errors are all about 10% of the corresponding mean R_{urea}. Thus, in general, the standard error of the mean effect of a treatment based on three sets of duplicate determinations should have a standard error of about 6% of the mean rate of urea synthesis. Some of the comparisons between different experiments reported in this paper differ more widely than would be expected from this analysis, but this is due to differences between animals, which can be quite large.

RESULTS

Relative effectiveness of ornithine and citrulline as catalysts of urea formation

We found, as Table 1 shows, that if 20 μmoles of urea is added to the 10 ml. of incubation medium it suppresses the urea formation in the absence of added catalyst almost completely, without appreciably affecting the rates of urea formation in the presence of added ornithine or citrulline.

This indicates that the effect of added catalyst is not properly measured by taking the difference between the rate in the presence of catalyst and the rate observed in the blank. We have made a practice in this work of using the addition of urea as a routine and disregarding any correction for the blank.

The results of a series of determinations of rates of urea synthesis in these conditions, in the presence of different concentrations of ornithine and citrulline, are shown in Fig. 2. The number by each point refers to the number of observations at each concentration of catalyst.

Table 1. *Effect of the addition of urea on the rate of urea synthesis by rat-liver slices*

About 100 mg. quantities wet wt. of liver in 10 ml. of medium were incubated at 38° for 30 min. in the presence of 100 μmoles of ammonia. R_{urea} = μmoles of urea/100 mg. dry wt. of tissue/hr.

Amount of urea added to 10 ml.	R_{urea} in the presence of		
	No catalyst	Ornithine (5·9 μmoles)	Citrulline (22·8 μmoles)
None	8·7 ± 0·54 (21)	16·9 ± 0·72 (11)	26·7 ± 1·13 (9)
20 μmoles	2·3 ± 0·27 (10)	16·4 ± 0·59 (18)	25·5 ± 0·91 (20)

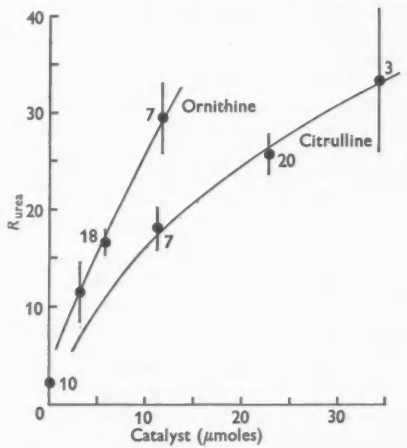

Fig. 2. Relation between catalyst concentration and rate of urea synthesis. Incubation for 30 min. of 100 mg. of liver in 10 ml. with 20 μmoles of urea and 100 μmoles of ammonia. Figures by points are numbers of observations. Vertical lines are 95% fiducial limits.

These data may be considered in two ways. In the first place, in the one instance in which nearly identical concentrations of the two catalysts can be compared (11·4 μmoles of citrulline and 11·8 μmoles of ornithine/10 ml.), the rate with ornithine as catalyst is highly significantly in excess of the rate with citrulline as catalyst, being roughly twice as great. In the second place, an R_{urea} in the region of 17 is obtained with 5·9 μmoles of ornithine/10 ml., but requires 11·4 μmoles of citrulline/10 ml., and the mean rates found with 22·8 and 34·2 μmoles of citrulline/10 ml. bracket the value found with 11·4 μmoles of ornithine/10 ml. Thus in the conditions of these experiments ornithine is roughly twice as effective as citrulline in catalysing urea synthesis.

Time course of urea synthesis

Nothing is known of the speed at which a steady state is attained in the synthesis of urea by liver slices. Experiments were therefore made with equimolar concentrations (11·4 μmoles/10 ml.) of ornithine and citrulline, and measuring the urea

Table 2. *Time course of urea formation*

Catalyst concentration was 11·4 μmoles/10 ml. in all experiments. Urea (20 μmoles) and ammonia (100 μmoles) were added initially to each tube.

Catalyst	Expt.	Urea formed (μmoles/100 mg. dry wt. of tissue)		
		0–15 min.	0–30 min.	0–45 min.
Ornithine	1	3·28	10·46	20·85
		3·29	9·54	15·97
	2	2·24	9·22	14·34
		1·98	8·95	13·96
Citrulline	3	3·54	5·17	10·27
		1·58	5·02	9·38
	4	1·94	7·90	11·48
		1·06	7·38	10·07

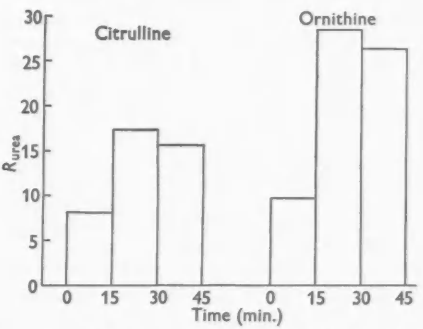

Fig. 3. Rates of urea synthesis in three successive 15 min. periods. The concentration of catalyst was 11·4 μmoles/10 ml. 100 mg. of liver, 20 μmoles of urea, and 100 μmoles of ammonia were used in all experiments. The figures are means of two duplicate experiments.

synthesized in 15, 30 and 45 min. periods. The data are given in Table 2, and the rates for the successive 15 min. periods derived from these data are shown in Fig. 3. An analysis of variance of the data in Table 2 yields estimates of the standard errors of the mean R_{urea} in each 15 min. interval. These are ± 3·43 for the ornithine series and ± 2·34 for the citrulline series. There is, therefore, no significant difference between the rates in the second and third 15 min. periods for either catalyst, and we may take the

mean rates over the 15–45 min. interval as estimates of steady-state rates. The analysis of variance yields a value of ±2·93 for the standard error of the difference between these steady-state rates for the two catalysts, and the observed difference is 10·82. The estimate of error is based on 18 degrees of freedom and the difference is therefore highly significant.

Since, as Fig. 3 shows, the mean rate in the first 15 min. is about half the steady-state rate for citrulline but only about one-third of the steady-state rate for ornithine, these observations indicate that the process catalysed by citrulline attains a steady state more rapidly than does the process catalysed by ornithine. However, the steady-state rate attained is only about half as great with citrulline as with an equimolar concentration of ornithine.

Another set of time-course observations was made in which a lower concentration of ornithine (5·9 μmoles/10 ml.) and a higher concentration of citrulline (22·8 μmoles/10 ml.) were compared. In this set it is again true that the rate in the first 15 min. is a distinctly larger fraction of the rate in the second 15 min. for citrulline than for ornithine. Thus over the range of catalyst concentrations that is of interest, it seems likely that the steady-state rate is always more rapidly attained with citrulline, the less effective catalyst. The results of this second set of observations are given in Fig. 4, together with a series of observations in which liver slices were incubated with ammonia for 15 min. before the addition of 5·9 μmoles of ornithine/10 ml. Although this pre-incubation with ammonia markedly increased the rate of urea synthesis it did not affect the lag in attainment of the steady-state rate: the ratio of mean rate in the second 15 min. to that in the first 15 min. was 1·61:1 when the slices were pre-incubated with ammonia and 1·67:1 in the absence of pre-incubation.

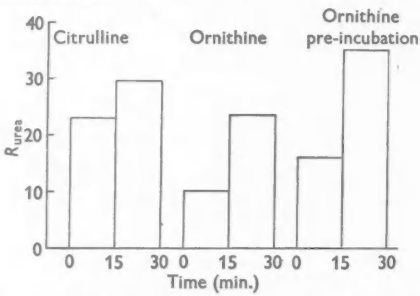

Fig. 4. Rates of urea synthesis in two successive 15 min. periods; 5·9 μmoles of ornithine and 22·4 μmoles of citrulline were used. 'Pre-incubation' means that ammonia was added 15 min. before the catalyst. Other conditions as in Fig. 3.

Effect of pre-incubation with ammonia on urea synthesis

Three sets of experiments were performed. In all sets the rate of urea formation over 30 min. periods was measured in controls and in the presence of ornithine (5·9 μmoles) or citrulline (22·8 μmoles), one set of observations being made in which catalyst and ammonia were added simultaneously and the reaction stopped at 30 min., whilst in the other set catalyst was added 15 min. after addition of ammonia, the reaction being stopped after a further 30 min. The control in this set was incubated with ammonia for 45 min., instead of 30 min. as in the first set. In the first series (series *A*), no urea was added to the medium. In the second series (series *B*), 20 μmoles of urea was added with the catalyst. In the third series (series *C*), urea was added 15 min. before the catalysts. The results of these experiments are given in Table 3. It will be seen that the effect of

Table 3. *Effect of pre-incubation with ammonia on the rate of urea synthesis in rat-liver slices*

In all experiments, liver slices were incubated for 15 min. before adding catalyst, and for 30 min. after adding it. P, addition of ammonia 15 min. before catalyst; N, addition of ammonia at same time as catalyst. In series *A*, no urea was added; in series *B* 20 μmoles of urea was added at the same time as catalyst; in series *C* 20 μmoles of urea was added at the beginning of incubation.

Series		R_{urea} in the presence of: No catalyst	Ornithine (5·9 μmoles)	Citrulline (22·8 μmoles)
A	P	15·2±0·67 (7)	31·7±2·06 (7)	31·7±4·82 (2)
	N	8·7±0·54 (21)	16·9±0·72 (11)	26·7±1·13 (9)
	P – N	6·5	14·8	5·0
	Difference from control	—	8·3	– 1·5
B	P	7·0±1·61 (3)	20·5±1·61 (3)	30·3±3·40 (3)
	N	2·0±0·51 (3)	16·9±0·96 (3)	28·8±3·83 (3)
	P – N	5·0	3·6	1·5
	Difference from control	—	– 1·4	– 3·5
C	P	3·3±2·23 (2)	15·5±0·36 (2)	22·7±2·90 (2)
	N	2·6±1·12 (2)	11·6±0·51 (2)	18·6±0·96 (2)
	P – N	0·7	3·9	4·1
	Difference from control	—	3·2	3·4

Table 4. *Ornithine disappearance, urea formation and changes in citrulline content of medium and liver*

Liver slices (about 100 mg.) were incubated for 30 min. in 10 ml. of medium containing 100 μmoles of ammonia, with and without ornithine.

Ornithine added (μmoles)	Ornithine disappearing (μmoles)	Citrulline found (μmoles)		Urea formed (μmoles)
		Medium	Liver	
None	Nil	0·72±0·051 (6)	0·22±0·028 (6)	5·47±0·49 (6)
5·9	1·52±0·073 (6)	0·60±0·062 (6)	0·16±0·023 (6)	10·40±1·03 (6)
Difference	1·52	−0·16	−0·06	4·93

Table 5. *Citrulline disappearance, urea formation and changes in ornithine content of medium and liver*

Liver slices (about 100 mg.) were incubated for 30 min. in 10 ml. of medium containing 100 μmoles of ammonia, with and without citrulline.

Citrulline added (μmoles)	Citrulline disappearing (μmoles)	Ornithine found (μmoles)		Urea formed (μmoles)
		Medium	Liver	
None	Nil	0·10±0·017 (6)	0·065±0·003 (6)	5·47±0·49
22·8	2·35±0·615 (4)	0·55±0·011 (4)	0·24 ±0·014 (4)	13·15±0·12
Difference	2·35	0·45	0·17	7·68

pre-incubation cannot be described simply. In the absence of urea, pre-incubation naturally increases the urea formation in the control since the liver slices are in contact with ammonia for a longer period. In these circumstances the effect of pre-incubation on the ornithine reaction catalysed by ornithine is much greater than can be accounted for by its effect on the control, but there is no greater effect on the reaction catalysed by citrulline than can be accounted for by effect on the control. Series *B* and *C* show that the longer the time for which added urea is present in the system, the less the effect of pre-incubation on the controls. Urea also abolishes the differences in response between the reaction catalysed by ornithine and the reaction catalysed by citrulline. The effects of urea in these experiments are of as much interest as those of pre-incubation with ammonia, since the urea concentration used was very low, being about 40 % of the physiological concentration in rat-tissue water.

Fate of ornithine and citrulline in liver slices

With methods available (Bronk & Fisher, 1956) for the estimation of ornithine and citrulline that are more specific than those of Chinard (1952) and Fearon (1939) we have used them to study the fate of these amino acids in liver slices.

Table 4 shows the changes in apparent citrulline content of slices and medium in the presence and absence of added ornithine, together with the amount of ornithine disappearing and the amount of urea formed. Table 5 gives the corresponding changes in apparent ornithine content of slices and medium in the presence and absence of added citrulline, together with the amount of citrulline disappearing and the amount of urea formed.

It has already been shown (Bronk & Fisher, 1956) that the amounts of preformed apparent ornithine and citrulline found in liver slices are not affected by prolonging the period of incubation from 15 to 30 min. On the other hand, the data of Tables 4 and 5 show that appreciable amounts of added amino acid are destroyed in 30 min., these amounts being 10–20 times as great as the total preformed apparent ornithine or citrulline in the slices. Thus the preformed apparent ornithine and citrulline behave quite differently metabolically from authentic amino acid added to the medium. This strengthens our previous conclusion that the apparent ornithine and citrulline found preformed in liver slices is contaminant material.

The data in Tables 4 and 5 give conclusive evidence that both ornithine and citrulline act catalytically in increasing the rate of urea synthesis. In both groups of observations more than three times as much urea was produced as amino acid lost.

The percentage of added ornithine disappearing (Table 4) is 26 % (1·52 out of 5·9 μmoles added), and the absolute amount disappearing is very little less than the amount of citrulline which disappears (Table 5) at a much higher initial concentration (2·35 out of 22·8 μmoles, or 10 %). These changes are large relative to the amount of contaminant material present and can be safely attributed to real changes in ornithine and citrulline content. Thus in both instances the mean concentration of catalyst does not change greatly from the initial value during an incubation period of 30 min., and citrulline probably disappears more slowly in comparable conditions than does ornithine.

In the presence of added ornithine (Table 4) there is no indication of an increase in the apparent citrulline content of the liver or medium. On the other hand, there is an increase in apparent ornithine content when citrulline is added (Table 5). However, this increase is small and cannot be attributed with certainty to a true increase in ornithine content.

Summation experiments with ornithine and citrulline

If the two catalysts participate in the same cycle, it is to be expected that, in a system not saturated with one catalyst, the addition of the other will increase the rate of urea synthesis. The same effect would be expected, but more markedly, amounting probably to an exact summation, if the two catalysts participated in completely independent cycles. However, as Table 6 shows, the rate of urea synthesis with the two catalysts present is lower in rat liver than the higher of the rates found with one of them alone. The first pair of experiments shown in this table was designed so that the higher rate was obtained with citrulline, the second pair so that the higher rate was obtained with ornithine. In both instances, the mean rate obtained with the two catalysts is intermediate.

Table 6. *Comparison of rates of urea synthesis in rat-liver slices in the presence of ornithine alone, of citrulline alone, and of ornithine and citrulline*

All incubations were for 30 min. in the presence of 20 μmoles of urea and 100 μmoles of ammonia. In Expts. 1 and 2, 5·9 μmoles of ornithine and 22·8 μmoles of citrulline were used. In Expts. 3 and 4, 11·8 μmoles of ornithine and 11·4 μmoles of citrulline were used.

	R_{urea} in the presence of		
Expt.	Ornithine	Citrulline	Citrulline and ornithine
1	17·4	21·3	21·2
	18·0	20·0	18·4
2	15·7	30·9	25·7
	15·9	28·3	24·7
Mean*	16·8±1·48	25·1±1·48	22·5±1·48
3	24·8	19·3	26·0
	23·6	17·4	20·1
4	32·5	18·1	24·1
	31·4	22·8	25·7
Mean*	28·1±1·31	19·4±1·31	24·0±1·31

* In both series of experiments there is a significant difference between the pairs of animals used. The standard errors of means given here are based on the residual variance, after eliminating effects of treatments and of difference between animals. In each series there are 8 degrees of freedom available for calculation of residual variance.

Table 7. *Comparison of rates of urea synthesis in guinea-pig liver in the presence of ornithine alone (5·9 μmoles), of citrulline alone (22·8 μmoles) and of citrulline and ornithine*

Incubations were for 30 min. in the presence of 20 μmoles of urea and 100 μmoles of ammonia.

R_{urea} in the presence of		
Ornithine	Citrulline	Ornithine and citrulline
15·5	15·7	15·0
15·1	18·1	14·4

Table 7 shows that in guinea-pig liver the rate with the two catalysts together is lower than with either alone. Although we have not made a full study of the relation between catalyst concentration and rate of urea synthesis for guinea-pig liver, the data in Table 6 suggest that ornithine is again the more effective catalyst here. With a citrulline concentration four times that of ornithine, the two rates of urea synthesis are the same.

DISCUSSION

The work described in this paper confirms the finding of Gornall & Hunter (1943) that ornithine is more effective than citrulline as a catalyst of urea synthesis in rat liver, and suggests that the same is true of guinea-pig liver. The conditions and technique of the present experiments differ from those of Gornall & Hunter at many points. Urea was added to our incubation media, our experiments lasted for 30 min. instead of 120 min., our procedure for estimating liver-slice weight gave a maximum estimate, and we used four times as much liver per unit volume of medium as did Gornall & Hunter. It is not surprising therefore that our estimates of activity of liver differ from theirs. Despite the many differences, the same relation between the activities of the catalysts appears.

The observations on time-course are of the greatest importance, since they effectively exclude hypotheses which seek to account for the greater effectiveness of ornithine on a basis of differential access to a single cycle. Whether the hypothesis takes the form of differential access to the interior of cells (which we have already excluded, Bronk & Fisher, 1956), differential access to intracellular inclusions, or differential rates of conversion into derivatives which are the effective catalysts, the expectations are: (1) that the difference in observed rates of urea synthesis will be caused by a greater lag in the attainment of the steady state in the reaction catalysed by citrulline; and (2) that when equimolar concentrations of the catalysts are used the same steady-state rate will be attained in both instances.

The data of Figs. 3 and 4 make it quite clear that the process catalysed by citrulline does not lag behind the one catalysed by ornithine in the approach to a steady state, and that the steady-state

rate with citrulline is of the order of half that attained with an equimolar concentration of ornithine.

The only other hypothesis which would permit one to retain the notion of a single cycle involving ornithine and citrulline is that the catalysts enter the cycle in the form of derivatives, and that free citrulline can be destroyed rapidly in liver cells. Tables 4 and 5 provide evidence on this point. They indicate that neither amino acid disappears very rapidly and that citrulline probably disappears more slowly than ornithine. Thus we may say that it is difficult, if not impossible, to account for the facts on a single-cycle hypothesis.

Pre-incubation with ammonia before addition of the catalyst produces an increase in the rate of the reaction catalysed by ornithine, but has no effect on the reaction catalysed by citrulline. It is difficult to see how this treatment could affect one catalysed process without affecting the other if ornithine and citrulline were directly concerned in the same cycle.

Against this background, the observation that ornithine and citrulline can mutually inhibit each other's catalysis of urea synthesis is positive evidence for two cycles. This phenomenon is inconsistent with any explanation of either catalytic process which involves, as an essential step, the conversion of either ornithine or citrulline into the other. Every revolution of a chemical catalytic cycle involves equally all of its chemical components, so that an increase in the rate of catalysis involves an increase in the concentration of all the components. In conditions such as those of the present experiments, in which it is possible to increase the rate of the reaction catalysed by ornithine by increasing the concentration of ornithine, there must be a corresponding increase in the concentration of all the components of the ornithine cycle. If citrulline, as such, were a component of the ornithine cycle, we should have this position: that increasing the concentration of citrulline by adding ornithine increased the rate of urea synthesis although, as experiment shows, increasing the concentration of citrulline by adding citrulline to ornithine decreases the rate of urea synthesis. There seems, therefore, to be no escape from the conclusion that citrulline itself is not a component of the ornithine cycle. Similar arguments indicate that ornithine is not a member of the citrulline cycle.

On the other hand, the same observations that lead to this conclusion point to a close relation between the two cycles. If the two were totally distinct the mutual inhibition observed in our experiments should not occur. It is also very difficult to believe that the mass of evidence for the partial reactions of the Krebs & Henseleit (1932) scheme which has been gathered in recent years (see for instance, Cohen, 1954; Krebs, 1952; Ratner, 1954) is without relevance to the nature of the cycles operating in the intact cell. Although we can adduce no specific evidence at the present juncture, we believe that all the observations could be reconciled if we were to postulate two cycles, each of the general nature of the Krebs & Henseleit (1932) cycle, but differing in that in both instances the catalysts ornithine and citrulline enter the cycle after a specific transformation. It is known that [α-*N*]-substituted arginine derivatives can be hydrolysed by arginase (Felix, Müller & Dirr, 1928; Hunter, 1938), so that if ornithine were to undergo transformation to one such derivative and if citrulline were to undergo some different transformation, then a basis could be found both for the anomalous ratio of effectiveness of the catalysts and for their mutual interference.

This type of explanation is shown diagrammatically in more detail in Fig. 5. In this figure the postulated two series of derivatives of ornithine, citrulline and arginine are shown as OX, CX, AX and OY, CY, AY respectively, and it is suggested that, whilst both ornithine and citrulline can enter each set, ornithine enters the one set more readily and citrulline the other. If ornithine enters the set having the higher natural rate of catalytic cycling, this will account for its greater effectiveness. The assumption that both ornithine and citrulline can compete for the two transforming enzymes, though only one of them can be transformed at a high rate by each enzyme, will mean that the net rate of transformation of the two, and therefore the total rate of entry of them into the cycles, will be reduced when both ornithine and citrulline are added to the system.

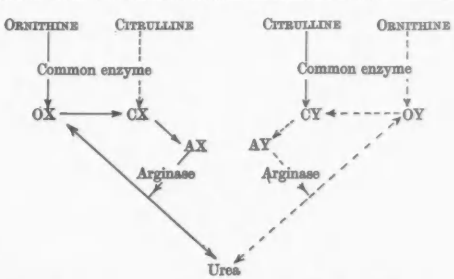

Fig. 5. Scheme of two catalytic cycles for formation of urea, devised to account for the greater effectiveness of ornithine and for the competition between ornithine and citrulline. Continuous lines indicate relatively rapid reaction; broken lines indicate relatively slow reaction. OX, CX, AX are one set of derivatives of ornithine, citrulline and arginine capable of interconversion into one another, and OY, CY, AY is another, similar set.

We suggest in Fig. 5 that arginase is common to the two cycles. Although the remaining steps of the two cycles are shown separately in this figure, there seems to be no objection to the assumption that the enzymes catalysing the remaining steps are common to the two cycles, provided that in physiological conditions these enzymes, as is the case with arginase, are likely to be far from the point of saturation. Thus provided that the enzymes of the partial systems that have been investigated in the last ten years are capable of transforming simple derivatives of ornithine and citrulline corresponding to our OX, OY, etc., this scheme provides a way of reconciling the whole body of work up to date on the mechanisms of urea synthesis.

SUMMARY

1. It has been confirmed that ornithine is a more effective catalyst of urea synthesis in rat liver than citrulline, and some evidence has been obtained that the same is true of guinea-pig liver.

2. Low concentrations of urea (2 mM) suppress urea formation in the absence of added catalyst and differentially affect the urea synthesis catalysed by ornithine and that catalysed by citrulline.

3. The greater effectiveness of ornithine is not due to a smaller susceptibility to destruction by liver.

4. Despite the greater effectiveness of ornithine, the reaction catalysed by ornithine reaches a steady state more slowly than the reaction catalysed by citrulline, and therefore measurements of mean rate of synthesis from the time of addition of catalyst underestimate the relative effectiveness of ornithine. The steady-state rate attained with ornithine is significantly higher than that attained with an equimolar amount of citrulline.

5. Incubation of liver slices with ammonia before adding catalyst has different effects on the reaction catalysed by ornithine and reaction catalysed by citrulline.

6. Ornithine and citrulline interfere with each other's catalytic reactions.

7. A new scheme of urea synthesis is proposed, based on these observations.

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The Relation of Ammonia Concentration to Urea Synthesis

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In the course of attempts to establish that the processes catalysed by ornithine and by citrulline which lead to urea synthesis in rat-liver slices were distinct, some experiments were made on the effect of variation of ammonia concentration on the rate of urea synthesis. These experiments were made in a restricted range of conditions, but it is not possible at present to extend the observations. The data show that the relation between ammonia concentration and rate of urea synthesis is more complex than has hitherto been thought.

We have also made observations on the relation between ammonia utilization by liver and the rate of urea synthesis in a variety of conditions. These experiments disclose a number of new features of ammonia metabolism in liver.

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METHODS

The methods used have been described in detail in the two previous papers (Bronk & Fisher, 1956*a*, *b*). Urea was estimated as NH_3 released by melon-seed urease, and the determination of free NH_3 was an essential part of this method. Thus, provided that the amount of NH_3 originally added to the incubation medium containing liver slices was known, NH_3 utilization could be computed from the data obtained during the urea determinations.

Glutamic acid and aspartic acid were separated from each other and from other amino acids by the paper-electro-

phoretic method described previously (Bronk & Fisher, 1956*a*), and determined by the ninhydrin-colorimetric method. Glutamine and asparagine could be distinguished from other ninhydrin-reacting substances on the electrophoresis papers, but the quantities of material present in the positions corresponding to these compounds were never large enough for reliable estimation.

The conditions in which the relation between NH_3 concentration and rate of urea synthesis were studied were as follows.

(1) 20 μmoles of urea/10 ml. was present in all experiments. We have already shown (Bronk & Fisher, 1956*b*) that this suppresses almost entirely urea synthesis from NH_3 in the absence of ornithine and citrulline, without affecting urea synthesis in the presence of the catalysts.

(2) The concentrations of catalyst were 5·9 μmoles of ornithine and 22·8 μmoles of citrulline in the 10 ml. of medium used.

(3) At the three higher concentrations (50, 100 and 200 μmoles/10 ml.) NH_3 was added initially as NH_4Cl in these concentrations, and the effective catalyst concentration was taken as the mean of this initial concentration and that found at the end of the experiment. At the lowest concentration of NH_3 used (25 μmoles/10 ml.), NH_3 was added initially to give this concentration, and a further equal amount of NH_3 was added half-way through the incubation period of 30 min. Again the mean of the initial and final concentrations, which in this case were nearly equal, was taken as the effective NH_3 concentration.

RESULTS

The results of these experiments are shown in Fig. 1. No estimates of variability are shown in this figure since the same data are shown in Table 1 (in terms of the ammonia equivalent of the urea formed), and standard errors of means are given there.

It appears that for both catalysts there is an increase in rate of urea synthesis with increasing ammonia concentration to some point between 25 and 50 μmoles/10 ml. (4·25–8·5 mg./100 ml.), and that thereafter the rate of urea synthesis declines with increasing ammonia concentration.

With the concentrations of catalysts used (5·9 μmoles of ornithine and 22·8 μmoles of citrulline/10 ml.), the rate of urea synthesis catalysed by ornithine is greater than the rate catalysed by citrulline at the lowest ammonia concentration, but lower at all higher ammonia concentrations.

Relation between ammonia utilization and ammonia concentration. It has long been known (Krebs, Eggleston & Hems, 1948) that when liver slices are incubated in conditions similar to those used in the present experiments more ammonia disappears than can be accounted for by the urea appearing. Table 1 shows that this is true of the experiments for which Fig. 1 shows the rates of urea synthesis. This table shows the ammonia utilization accounted for by urea synthesis and that not so accounted for, which we have called the extra ammonia utilization. It will be seen that extra ammonia utilization is dependent on ammonia concentration, but in the opposite way to that in which urea synthesis is dependent on it. Whenever there is an increase in

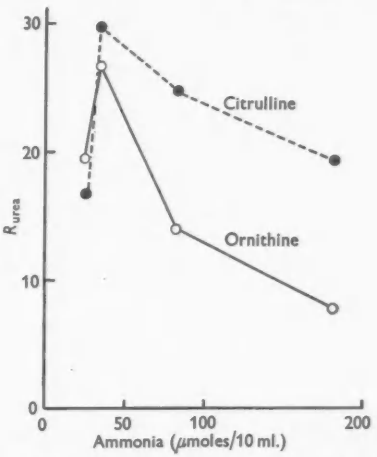

Fig. 1. Relation between the rate of urea synthesis and ammonia concentration, with about 100 mg. of liver slices, incubated in 10 ml. of medium containing 20 μmoles of urea for 30 min., in the presence of 5·9 μmoles of ornithine or 22·8 μmoles of citrulline. Each point represents the mean of three duplicate determinations.

Table 1. *Relation between ammonia utilization in liver slices and ammonia concentration*

Each figure is the mean of three observations. Urea (20 μmoles) was added in all cases and the initial ammonia content of each incubation tube was 100 μmoles. The incubation time was 30 min.

Initial ammonia concn. (μmoles/10 ml.)	Ammonia utilization (μmoles/100 mg. dry wt./hr.) due to urea formation (urea) and to other processes (extra)					
	In presence of ornithine (5·9 μmoles)			In presence of citrulline (22·8 μmoles)		
	Urea	Extra	Total	Urea	Extra	Total
25	39·0±2·50	16·4±3·31	55·4	33·6±0·98	13·1±1·10	46·7
50	53·4±3·02	10·1±3·22	63·5	59·4±4·64	5·9±3·25	65·3
100	28·0±4·42	45·0±1·68	73·0	49·8±3·36	53·5±10·5	103·3
200	15·6±2·98	59·5±9·66	75·1	39·2±7·90	62·0±6·52	101·2

Table 2. *Effect of change of ornithine and citrulline concentration on ammonia utilization*

All incubations were for 30 min. in the presence of 20 μmoles of urea and 100 μmoles of ammonia. Numbers in parentheses indicate numbers of observations.

Catalyst	Concn. (μmoles/10 ml.)	Ammonia utilization (μmoles/100 mg. dry wt./hr.) due to urea formation (urea) and to other processes (extra)		
		Urea	Extra	Total
Ornithine	2·95	22·8±1·48 (3)	40·7±3·50 (3)	63·5
	5·9	32·8±1·18 (18)	36·1±2·05 (18)	68·9
	11·8	58·6±2·90 (7)	25·9±4·24 (7)	84·5
Citrulline	11·4	35·8±1·78 (7)	37·7±2·81 (7)	73·5
	22·8	51·0±1·82 (20)	30·3±1·98 (20)	81·3
	34·2	66·0±3·50 (3)	22·8±4·65 (3)	88·8

Table 3. *Effect of urea on ammonia utilization*

All incubations were for 30 min. in the presence of 100 μmoles of ammonia. Numbers in parenthese indicate numbers of observations.

Catalyst	Urea added (μmoles)	Ammonia utilization (μmoles/100 mg. dry wt./hr.) due to urea formation (urea) and to other processes (extra)		
		Urea	Extra	Total
None	0	17·4±1·08 (21)	27·2±2·41 (21)	44·6
	20	4·6±0·54 (10)	48·3±2·20 (10)	52·9
Ornithine (5·9 μmoles)	0	33·8±1·44 (11)	15·3±1·61 (11)	49·1
	20	32·8±1·18 (18)	36·1±2·05 (18)	68·9
Citrulline (22·8 μmoles)	0	53·4±2·26 (9)	22·4±3·50 (9)	75·8
	20	51·0±1·82 (20)	30·3±1·98 (20)	81·3

Table 4. *Rates of extra ammonia utilization in successive 15 min. periods*

Each figure is the mean of three observations. Incubations were for 30 min. in presence of 20 μmoles of urea and 100 μmoles of ammonia.

Catalyst	Extra ammonia utilization (μmoles/100 mg. dry wt./hr.)	
	0–15 min.	15–30 min.
Ornithine (5·9 μmoles)	52·5±0·81	16·1±1·10
Citrulline (22·8 μmoles)	60·0±4·65	17·4±1·12

rate of urea synthesis there is a decrease in extra ammonia utilization, and vice versa.

Relation between catalyst concentration and ammonia utilization. Table 2 gives a summary of the relation between ornithine and citrulline concentration on the one hand and ammonia utilization on the other. Once again, it is clear that there is a more or less reciprocal relation between ammonia utilization for urea synthesis and extra ammonia utilization. All the experiments reported here were carried out in the presence of added urea.

Effect of urea on ammonia utilization. Table 3 shows that, in contrast to the data already presented, the addition of urea increases the rate of extra ammonia utilization, without any corresponding effect on the formation of urea. This is striking, particularly in view of the very low concentration of urea used, which corresponds to 12 mg./100 ml., and is considerably lower than the physiological concentration in the body water of the rat (about 30 mg./100 ml.).

Time course of extra ammonia utilization. We have made a few observations on the time course of extra ammonia utilization which indicate that it falls off rapidly with time. These observations are reported in Table 4.

Fate of the extra ammonia used. Krebs *et al.* (1948) have shown with homogenates that some but not all of the ammonia disappearing is converted into glutamic acid. We have measured glutamic acid and aspartic acid in liver slices and medium by the techniques of desalting with resins, concentration, paper electrophoresis, and colorimetric estimation of ninhydrin colour as the copper complex, which have been described in a previous paper (Bronk & Fisher, 1956*a*). Table 5 compares the extra ammonia utilization and the dicarboxylic acid formation in liver slices in the absence of catalyst with the findings in the presence of ornithine or citrulline. These experiments were performed without added urea but with the pre-incubation of slices with ammonia for 15 min. before addition of catalyst. The total incubation time in all instances was 45 min.

It will be seen that dicarboxylic acids found are equivalent to between 5 and 10% of the extra ammonia utilization. The extent of dicarboxylic acid formation from added ammonia is less than

Table 5. *Effects of ornithine and citrulline on the glutamic acid and aspartic acid contents of incubated liver slices*

Each figure is the mean of three observations. Incubations were for 45 min. in the presence of 100 μmoles of ammonia, the catalyst being added at 15 min.

Catalyst added	Extra ammonia utilization (μmoles)	Dicarboxylic acid found (μmoles)		
		Glutamic	Aspartic	Total
None	27·7	1·03	1·70	2·73
Ornithine (5·9 μmoles)	22·0	0·81	1·04	1·85
Citrulline (22·8 μmoles)	22·2	0·73	0·33	1·06

this indicates, since about 0·5 μmole of dicarboxylic acid is found when slices are incubated without added ammonia. Inspection of the electrophoresis papers shows that although traces of glutamine and asparagine are present, they cannot account for any significant fraction of the extra ammonia utilization.

Clarkson & Kench (1956) have recently shown that as much as 36% hydrolysis of glutamine can take place on a cation-exchange resin (Zeo-Karb 225). This introduces some uncertainty into the interpretation of our measurements, but it still remains that the proportion of extra ammonia utilization which could be accounted for as dicarboxylic acids and their amides could not exceed 10–20%, even if one supposed that all of the dicarboxylic acid found had been derived by resin hydrolysis from liver acid amides.

DISCUSSION

These experiments throw some light on the discrepancy between the observed rate of urea synthesis in liver slices and the rate necessary to account for physiological urea production. One of us (Fisher, 1954) has suggested that a mean rate of urea synthesis corresponding to about 36 μmoles of urea formed/100 mg. dry wt. of tissue/hr. (R_{urea}) is necessary to account for the average rate of protein metabolism in the rat and that, owing to the non-uniformity in the rate of protein catabolism, the peak capacity must be much higher than this.

The existing data (Krebs & Henseleit, 1932) suggested that the rate of urea synthesis of liver slices fell off progressively as the ammonia concentration fell below 200 μmoles/10 ml., and the rate observed at an initial ammonia concentration of 3·18 mg./100 ml. (19 μmoles/10 ml.) and in the presence of 7·6 μmoles of ornithine/10 ml. corresponded to an R_{urea} of 18. But there was no added urea in these experiments, as there would be in physiological conditions, and we have shown (Bronk & Fisher, 1956*b*) that added urea almost completely suppresses the urea production in the absence of added catalyst. It appears from Krebs & Henseleit's data that the R_{urea} in the absence of ornithine would be in the region of 13–15. Such calculations made it appear that the process studied by these workers could not be responsible for physiological urea synthesis, which must be taking place at a much higher rate at a much lower ammonia concentration.

The present data suggest much higher rates at moderately low ammonia concentration ($R_{urea} = 19$ at a mean ammonia concentration of 25 μmoles/10 ml., which is 4·25 mg./100 ml.). A large part of the difference between these sets of observations is accounted for by the fact that recalculation shows that in Krebs & Henseleit's (1932) work the rate of urea synthesis was limited by the supply of ammonia.

Our figures are still too low to account for physiological urea formation, but in this work we have been measuring mean rates from the time of addition of catalyst, and we have shown (Bronk & Fisher, 1956*b*) that the reaction catalysed by ornithine, which is the more probable physiological pathway (see below), does not attain a steady state until the second 15 min. period of our observations. In the constant presence of ornithine *in vivo* the rates of urea synthesis at given ammonia concentrations could be 1·5 times as high as those we have found during the 0–30 min. period.

We base our belief that the reaction catalysed by ornithine is the more likely to be of physiological importance on the data of Fig. 1. In these experiments, the concentration of citrulline was regularly approximately four times that of ornithine. We may measure relative effectiveness roughly by the ratio of R_{urea} to catalyst concentration (Bronk & Fisher, 1956*b*). The relative effectiveness in these terms will depend to some extent on what concentrations of the two catalysts are being compared. But if we make experiments in which a given concentration of ornithine is constantly being compared with a given concentration of citrulline, variations in relative effectiveness can be taken as showing reliably the differential effect of the experimental variable on the two catalysed reactions. The effect of change in ammonia concentration on the relative effectiveness of the two catalyses is shown in Table 6, which is derived from the data of Fig. 1. This table shows a sharp rise in relative effectiveness of ornithine as the ammonia concentration falls, and suggests that at physiological concentrations of ammonia the cycle catalysed by ornithine is likely to be far the more important. The point already made about the relation between the rates of urea synthesis measured over 30 min. and the steady-state rates in the constant presence of catalyst comes in again here.

Table 6. *Relative effectiveness of ornithine and citrulline as catalysts of urea formation*

Effectiveness is measured as R_{urea}/μmole of catalyst added. Incubations were for 30 min. in the presence of 20 μmoles of urea and 100 μmoles of ammonia.

Ammonia concn. (μmoles/10 ml.)	Catalytic effectiveness of ornithine relative to citrulline
25	4·59
50	3·57
100	2·17
200	1·54

The measured rates of the reaction catalysed by ornithine are further from the steady-state rates than the measured rates of the reaction catalysed by citrulline, so that the steady-state relative effectiveness of ornithine may be expected to be still higher than the observed relative effectiveness.

All that is certain about ammonia utilization by liver slices is that a large fraction of it is not accounted for as urea or as dicarboxylic acids or their amides. It is probable, however, that the unknown processes of ammonia utilization are not quantitatively important. On the whole, they occur to a greater extent the lower the rate of urea synthesis, so one might expect that when better conditions for urea synthesis were discovered these processes would be largely suppressed. Since, in addition (Table 4), extra ammonia utilization falls off very sharply with time, the processes involved probably do not proceed at any great rate in physiological conditions.

The relation between ammonia metabolism and urea synthesis is clearly complex. On the one hand, increasing the ammonia concentration beyond a certain point decreases the rate of urea synthesis and increases the rate of utilization of extra ammonia. At low concentrations of ammonia, at which urea synthesis is diminished, extra ammonia utilization is increased. Also, the rate of urea synthesis increases with time and the rate of extra ammonia utilization decreases with time. But this simple reciprocal relation is complicated by the observation (Bronk & Fisher, 1956*b*) that pre-incubation of liver slices with ammonia increases the rate of urea synthesis. We have examined the possibility that the effect of pre-incubation is due simply to a lowering of the ammonia concentration before adding the catalyst. The diminution in ammonia concentration that would be necessary at an initial ammonia concentration of 100 μmoles/10 ml. is of the order of 50 %, whereas the observed diminution in ammonia concentration during pre-incubation is 10–15 %. There must presumably therefore be products of ammonia metabolism which favour urea synthesis as well as products which interfere with it.

SUMMARY

1. The rates of urea synthesis by rat-liver slices, catalysed by ornithine and by citrulline, increase with increasing ammonia concentration up to about 50 μmoles of ammonia/10 ml. and then fall off.

2. The effectiveness of ornithine as a catalyst increases relative to the effectiveness of citrulline as the ammonia concentration falls.

3. The effects of ammonia, catalyst and urea concentration on ammonia utilization have been studied. In general, urea formation and extra ammonia utilization vary reciprocally.

4. Only about 5–10 % of the extra ammonia utilization is due to formation of dicarboxylic acids or their amides.

5. On the basis of this and other work it is suggested that the cycle catalysed by ornithine is the more likely of the two cycles postulated by Bronk & Fisher (1956*b*) to be responsible for physiological urea synthesis.

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Changes in the Cell Wall of the Pear During Ripening

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The cell wall is commonly regarded as an inert background to the metabolism of the plant, although it has been known from the time of Schulze (1892) that cell-wall polysaccharides are used as reserve food materials by many germinating seeds. The ripening of stored fruit is another instance of rapid physiological change in an isolated, non-assimilating tissue, and Barnell (1943) has found a rapid depletion of the hemicelluloses of the ripening banana. Similar but less drastic changes have been reported for the pomaceous fruits by various authors (Smith, 1935; Kidd & West, 1939; Kidd, West, Griffiths & Potter, 1940; Echevin, 1941).

Studies on changes in the cell wall are often difficult to interpret because of the uncertain nature of the fractions which are extracted from it by conventional procedures. 'Pectin', 'hemicellulose' and 'α-cellulose' are the resultants of certain sets of operations rather than chemical individuals. Each of these fractions is normally a mixture of polysaccharides that it is difficult to fractionate further, and the same polysaccharide may be present in more than one fraction.

Hydrolysis of any of these fractions may give a mixture containing as many as seven sugars, and although it is not possible to separate quantitatively the original mixture of polysaccharides, the mixture of sugars can be separated on a paper chromatogram (Jermyn & Isherwood, 1949; Isherwood & Jermyn, 1951) and each estimated quantitatively. If one assumes that each sugar is produced by the hydrolysis of a single polysaccharide, e.g. that all the xylose is obtained originally from xylan no matter which fraction is hydrolysed, then the total amount of any one polysaccharide can be calculated from the amount of the corresponding sugar in the hydrolysis products of the various fractions. This method is open to the criticism that the polysaccharides may contain more than one sugar, but in the pear it has been definitely established that the bulk of the glucose can be accounted for as cellulose, and of the xylose as xylan. These two polysaccharides were isolated in good yield (calculated in terms of the corresponding sugars produced by hydrolysis) from pear cell wall. Pectic acid is known to consist, mainly, if not entirely, of galacturonic acid residues, and araban and galactan of arabinose and galactose residues, respectively (Hirst, 1942; Hirst & Jones, 1938, 1939; Beavan, Hirst & Jones, 1939). The only other sugar present in any amount, mannose, represented only a small proportion of the whole. With the pear cell wall the assumption seems justified that changes in the polysaccharide pattern of the cell wall can be followed by analysing the mixture of sugars produced on hydrolysis. In the present study these methods are applied to give complete analyses of the cell wall of the ripening pear in terms of the polysaccharides corresponding to individual sugar residues.

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MATERIALS

Conference pears grown at East Malling Research Station, Kent, and received during September 1948, were mixed after any damaged or defective fruit had been rejected.

In the main experiment, twelve lots of 50 pears of about the same weight were chosen at random. Each lot was weighed and then the individual pears were placed out of contact with each other on trays lined with waxed Viscose film. The trays were placed on the floor of a light-proof room at +15° and the samples ripened under as nearly identical conditions as possible. The pears were examined at six successive stages, two lots of 50 pears being separately analysed at each stage and the results treated as duplicates. The progress of ripening was assessed by the change in colour of the skin (green to yellow) and by the firmness of the flesh. The latter was measured by the use of an instrument (Penetrometer) which recorded the force (kg.) required to drive a plunger (circular cross section, diameter 1 cm.) into the intact pear.

In subsidiary experiments (cf. Tables 5, 6 and 8) the experimental conditions were varied somewhat from those described above though the general conditions were similar. Any slight variations are mentioned appropriately in the text.

A description of the pears taken at each stage in the main experiment is given in Table 1.

METHODS

Preparation of cell-wall material

Duplicate batches of pears at the appropriate stage in the maturation of the fruit were removed from storage, weighed, and any rotten fruit removed. A correction was applied for

Table 1. *Properties of pears stored at +15° in the dark*

Each lot contained 50 pears, two lots being treated as duplicates. The 'Penetrometer' reading (see text) measured the firmness of the flesh.

Mean wt. per pear in each lot (g.)	Days at +15°	Condition	'Penetrometer' reading (kg.)	Loss of wt. during storage, calc. on original wt. of fruit (%)
161·5	Initial	Green	8·6	—
159·2	—	—	8·7	—
145·1	7	Short of ripe	3·1	4·4
170·5	—	—	3·8	4·5
151·7	11	Yellow, just ripe	2·9	2·6
168·8	—	—	2·8	4·4
165·9	16	Overripe	1·9	4·2
152·0	—	—	2·1	6·1
163·8	23	Sleepy	—	6·3
153·4	—	—	—	10·0
159·1	31	Slushy	—	10·0
158·5	—	—	—	11·2

the loss of material due to the removal of these fruit. The remainder of the fruit in each batch were peeled, cored, frozen overnight at −20° and finally ground to a flour in a rotary rasp, the temperature never rising above about −5°. The weight of dressed material varied between 65 and 75% of the initial weight of the pears, the lower figures being obtained with overripe pears which were difficult to peel satisfactorily. These were peeled over a large vessel so that the expressed juice could be added to the main bulk of the material. The frozen flour was stored in sealed containers at −25°, and before analysis was thoroughly mixed. The frozen flour (500 g.) was added to 2 l. of boiling ethanol and the whole brought to the boil as rapidly as possible and maintained at the boiling point for 30 min. The mixture was filtered through a sintered-glass funnel, and the residue extracted with several changes of boiling 95% (v/v) ethanol until the filtrate gave a negative anthrone test for carbohydrates (Dreywood, 1946). The final product was dried in a current of air. Yield, 15–18 g. from each batch.

The cell-wall material prepared in this way contained all the polysaccharides originally present in the cell sap. These were mostly members of the pectic group of substances.

Fractionation of cell-wall material

To provide an overall picture of the analysis to which each sample of cell wall was subjected, a typical example is given in Table 2.

The ethanol-insoluble polysaccharides were first fractionated by extracting all the material soluble in hot water (this can be regarded as roughly equivalent to the 'pectin' of earlier workers). The insoluble residue was delignified by treatment with sodium chlorite, and the polysaccharides remaining ('holocellulose') were fractionated by extracting with N- and 4N-KOH.

This left a final residue of polysaccharides which were not fractionated further ('α-cellulose'). At all stages, the various products were analysed for ash, nitrogen, methoxyl and lignin, and, after hydrolysis, for their constituent sugars. The results were summed so as to obtain a comprehensive picture of the composition of the cell wall and to detect the presence of unknown compounds.

The details of the various extraction and hydrolysis procedures are as follows.

Material soluble in hot water. The cell-wall preparation (10 g.) was extracted with 750 ml. of water at 98–100° for 12 hr. in a boiling-water bath. The mixture was filtered through a sintered-glass funnel and the residue washed with 6 × 200 ml. of boiling water. The residue was thoroughly mixed with acetone and washed on the filter with more acetone. It was then dried in a current of air. Yield of insoluble material, 6·5–7·5 g. Addition of such salts as ammonium oxalate to the extracting liquor did not lead to any significant increase in the amount of material removed.

The filtrate and washings from the extraction described above were concentrated under reduced pressure, at a maximum bath temperature of 40°, to a final volume of 75 ml. The polysaccharides were not precipitated from this concentrate by the addition of ethanol because earlier experiments had shown that the araban component is extensively hydrolysed by the extraction procedure and would not be precipitated. The whole concentrate was therefore used for the subsequent analysis.

Holocellulose. The insoluble residue remaining after the water-soluble material had been extracted was delignified by the method described by Wise, Murphy & D'Addieco (1946) for wood.

The material (5 g.) was suspended in 160 ml. of water in a 250 ml. Erlenmeyer flask, 10 drops of acetic acid and 1·5 g. of sodium chlorite were added and the flask was immersed to a point above the level of the liquid inside in a water bath at 75°. The flask was closed by a loose-fitting glass bubble. At hourly intervals 10 drops of acetic acid and 1·5 g. of sodium chlorite were added. After treatment for 4 hr. (the lignin content had fallen from 16% to 2–3%) the contents of the flask were filtered through a small sintered-glass funnel. The flask was washed with several portions of ice water to remove adhering matter, and the contents of the funnel were washed repeatedly (about 10 times) with ice water, and then with acetone. The powder was dried in a current of air. The yield of holocellulose appeared to increase as delignification proceeded. A typical experiment is described in Table 3. The apparent rise of about 5% in the amount of the holo-

Table 2. *Fractionation of cell-wall material from Conference pears ripened for* 16 *days at* +15°

Figures are calculated as percentage of initial wt. of alcohol-insoluble material. Sugars are expressed in the anhydro form.

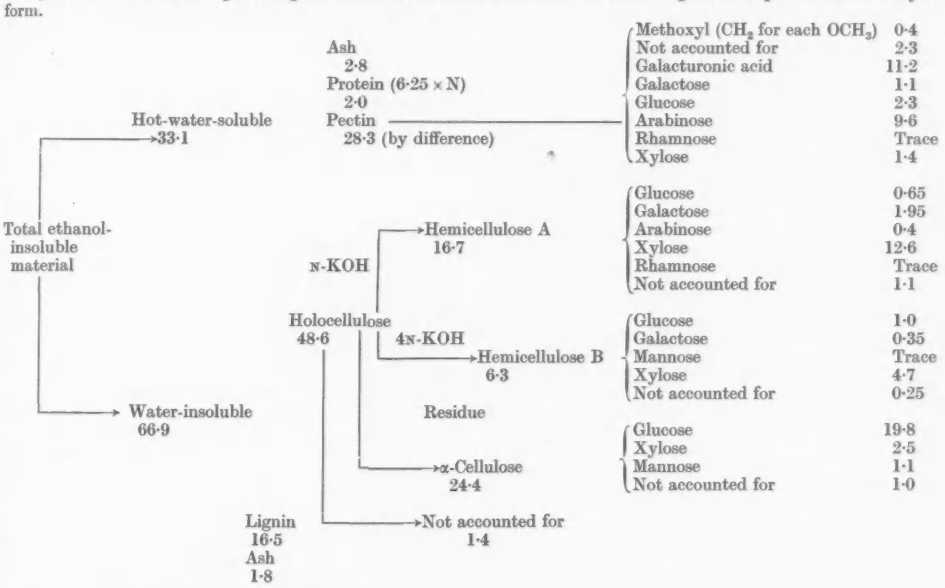

Table 3. *Yield of holocellulose from pear cell wall*

Alcohol-insoluble material treated with sodium chlorite (Wise *et al.* 1946) to remove lignin (see text).

Time of extraction (hr.)	Yield of delignified residue (%)	Lignin remaining in residue (%)	Apparent holocellulose (cell wall less lignin) (%)
0	100	24·5	75·5
1	95·0	16·4	78·6
2	90·4	10·7	79·7
4	82·1	2·1	80·0

cellulose occurred in all the samples examined. It seems to be due to the inherent defects of hydrolytic methods of estimating lignin. These lead to condensations between lignin and various substances in the cell wall (especially protein) and result in too high a figure for lignin.

The extraction liquors from the procedure described above gave only very faint tests for carbohydrate by the sensitive anthrone method (Dreywood, 1946) so that the loss of polysaccharide material was negligible.

Hemicellulose A. The holocellulose was fractionated according to the method of Wise *et al.* (1946) with the modification that the alkali extractions were not carried out under a stream of N_2. It was found that if the extractions were made in small Erlenmeyer flasks filled very nearly to the top with the extracting solution, the modification did not affect the results and the procedure was more convenient when a large number of analyses had to be made.

The holocellulose (2·5 g.) was placed in a 125 ml. Erlenmeyer flask and 100 ml. of N-KOH added. The flask was closed with a rubber bung and placed at a constant temperature of 20°. The contents were swirled gently at 10 min. intervals for 2 hr. The mixture was filtered through a sintered-glass funnel, washed with 50 ml. of N-KOH and then 150 ml. of water. The filtrate and washings were run into a suction flask, containing 10 ml. of acetic acid. This solution was transferred to a 2 l. flask and the suction flask rinsed twice with 25 ml. of water. The combined filtrates and washings were then diluted to 2 l. with ethanol and allowed to stand overnight.

The white flocculent precipitate was centrifuged, and then washed on the centrifuge successively with ethanol, acetone and ether. If the dehydration had been carried out properly the product was a light fluffy powder. This contained about 80% of the lignin that was originally present in the holocellulose.

Hemicellulose B. The residue after the extraction of the hemicellulose A was returned to the 125 ml. Erlenmeyer flask and 100 ml. of 4N-KOH added. The flask was placed at a constant temperature of 20° and swirled gently at 10 min. intervals for 2 hr. After the treatment, the liquid was filtered through a sintered-glass funnel and the residue washed with 25 ml. of 4N-KOH, 250 ml. of water and 25 ml. of 2N acetic acid. The filtrate and washings were run into a suction flask containing 45 ml. of acetic acid. The hemicellulose B was isolated from this solution in the same manner as for hemicellulose A described above.

α-Cellulose. This was the residue after the extraction of the hemicellulose B.

Hydrolysis of cell-wall fractions

Material soluble in hot water. The concentrated extract (75 ml.), to which had been added about 0·05 g. of urea nitrate, was heated in a boiling-water bath under reflux. When the temperature of the solution reached that of the bath, 3 ml. of colourless HNO_3 (sp.gr. 1·57) was added slowly with shaking. It was important not to add the acid till the contents of the flask were hot, as otherwise a considerable amount of the polysaccharide material precipitated and only redissolved after protracted heating. The acidified extract was heated for 12 hr. in the boiling-water bath, and then cooled. Tests showed that the last trace of digalacturonic acid disappeared after hydrolysis for 12 hr., though the maximum yield of galacturonic acid was produced after 10 hr. and was about 2% higher than the value at 12 hr. The recoveries of various sugars treated in the same way were arabinose 92%, galactose 97%, and glucose 96%. The recovery of galacturonic acid by this method, compared with that produced by total hydrolysis with a polygalacturonidase from the mould *Botrytis*, showed that 91 and 89% were produced at 10 and 12 hr. respectively (Jermyn & Tomkins, 1950).

The recoveries of the aldose sugars in the presence of galacturonic acid were rather less than the figures given above, but not by any definite amount. It was found in practice that the sugars recovered from the hydrolysis of the water-soluble fraction averaged about 90% of the amount expected, but because the recoveries of the various sugars were not sensibly constant no attempt was made to apply correction factors. The results for this fraction will therefore be low by about 10%.

Hemicelluloses. The hemicellulose fraction (400 mg.) was mixed with 10 ml. of 0·5 N-HNO_3 containing a few crystals of urea nitrate in a test tube closed with a glass bubble and the whole heated in a boiling-water bath for 4 hr. With hemicellulose A a little undissolved material remained, but tests showed that this was not carbohydrate and that it was probably connected with the lignin known to be present in the original material.

α-Cellulose. To 500 mg. of α-cellulose was added 5 ml. of 72% (w/w) H_2SO_4 in 0·5 ml. portions, the mixture being thoroughly stirred after each addition. After standing at room temperature for 2 hr., the α-cellulose dissolved completely. The solution was then poured into 360 ml. of water (including any washings necessary to effect a quantitative transfer of the solution) and the whole boiled under reflux for 12 hr. At the start the liquid was often faintly opalescent, but this soon disappeared, and at the end the amount of material remaining undissolved was less than 5 mg. and consisted almost entirely of ash. A few drops of a 1% solution of methyl red were added and then 15 g. of $BaCO_3$. The mixture was heated in a boiling-water bath, with constant shaking, until it was neutral to methyl red, and then filtered while hot through a sintered-glass funnel. The barium precipitate was washed exhaustively with boiling water (6 × 50 ml.) and the combined filtrate and washings were evaporated *in vacuo* to about 15 ml. A few drops of 0·1 N-H_2SO_4 were added at the start of the distillation and an occasional addition was made subsequently to keep the solution neutral to methyl red. This was found necessary to prevent the isomerization of any aldopentoses into ketoses in the slightly alkaline (pH 8) solution left after neutralization with $BaCO_3$. Xylose and arabinose are particularly susceptible.

Analysis

Sugars in the hydrolysis products. These were separated and estimated by the method described earlier by Jermyn & Isherwood (1949) with a paper chromatogram. An important improvement to this method has recently been described by Wager (1954). The hydrolysate from the α-cellulose was used directly but the nitric acid in the other hydrolysates (hemicellulose and water-soluble material) had to be removed before they could be examined on a paper chromatogram. The general procedure was as follows. The solution of sugars, after hydrolysis, was neutralized with 2 N-KOH and evaporated to dryness *in vacuo*. A mixture of absolute ethanol and benzene was distilled through the residue to remove any traces of water remaining, and the thoroughly dried residue was extracted with absolute ethanol (in which KNO_3 is almost insoluble) until all reducing material had been removed. The alcoholic solution was decolorized with a little charcoal, and evaporated to dryness *in vacuo*. With the hydrolysates from the water-soluble material (pectin) a slight modification was used. The dried residue was extracted with acetic acid instead of ethanol, filtered from suspended KNO_3 and the sugars were precipitated by the addition of anhydrous ether (5 vol.). The syrupy precipitate was redissolved in acetic acid and again precipitated with ether. The residue of sugars obtained by either method was dissolved in an appropriate volume of water to give a 2% solution suitable for chromatography.

Compounds other than polysaccharides in the cell wall. These were determined on each fraction and the figure for the carbohydrate content was corrected appropriately. There is almost certainly some error involved in this procedure. It is known, for example, that the water-insoluble protein present in the cell-wall material is destroyed by chlorite oxidation, and in some cases contributes as much as 10–20% to the apparent lignin figure. A similar state of affairs is probably true of other constituents, so that the corrected figure for the carbohydrates can be regarded only as approximate. The following constituents were determined: lignin (Ritter, Seborg & Mitchell, 1932), methoxyl (Hickinbottom, 1936), nitrogen ((Kjeldahl) Markham, 1942), volatile materials by estimating the loss in weight after drying at 105° for 12 hr., and ash by incineration at red heat.

Typical analysis. The figures obtained for the various sugars produced by the hydrolysis of the cell wall were recalculated to give the amounts of anhydro sugars originally present in the cell wall. The anhydro sugar (hexose or pentose minus one molecule of water) was regarded as being the equivalent of the appropriate polysaccharide. The figures were all based on the original fresh weight of the pears, and the anhydro sugars were summed irrespective of the fraction of the cell wall examined. The only exception was the glucosan removed by hot water; this has been treated as if it were entirely starch, and has been excluded from the cell-wall totals. There is almost certainly some error in this procedure but the results can be compared with those of other workers (Kidd *et al.* 1940) who determined the cell wall of pear as 'alcohol-insoluble material other than starch', and recorded loss of weight after treatment with a crude diastase preparation as a measure of starch content. Two assumptions have been made in calculating the amounts of the various polysaccharides in the original fresh fruit from the amounts of the anhydro sugars in the derived cell wall.

Perhaps the most important is that the flesh and skin removed with the peel and cores was identical with that retained in the dressed fruit. An analysis of a sample of pear peelings compared with the corresponding analysis on the dressed fruit is given in Table 4. The figures show that the composition is very much the same, any observed differences being of less consequence in practice because the peelings form only 15–20% of the total weight of the fruit. The other assumption was that the loss in weight during storage was uniformly distributed throughout the fruit.

RESULTS

Qualitative and quantitative composition of the alcohol-insoluble polysaccharides

The sugars produced on hydrolysis of all the arbitrary fractions—pectin, hemicellulose A and B, and α-cellulose—were glucose, xylose, arabinose, galactose, galacturonic acid and rhamnose (a trace). The rhamnose, for which no analytical figures are given, was never present in any fraction in more than trace amounts, and it formed at the most perhaps 1% of some pectin fractions. A uronic acid could be shown to be present in the hemicellulose fractions but could not positively be identified on the paper chromatograms. Examination of a purified xylan prepared from these fractions (Chanda, Hirst & Percival, 1951) showed that it contained one glucuronic acid terminal unit to a chain of 115 xylose units, and the inference is that the uronic acid present in the hemicellulose fractions was probably glucuronic acid.

A study of the composition of each of the arbitrary fractions throughout the storage of the fruit showed that there were no consistent ratios between the sugar residues present, such as would have been expected if the fractions represented definite components of the cell wall. It appears that each stage of the extraction procedure removes from the cell wall a complex mixture of polysaccharides, the composition of which is governed by the previous history of the sample. An illustration of this is given in Table 5.

Table 4. *Analyses of the ethanol-insoluble polysaccharides from the combined peelings of 200 pears*

The pears (mean wt. 160 g.) had been stored for 11–23 days at +15° and were just ripe to overripe in condition. The figures are compared with those for dressed pears (mean wt. 165 g.) which had been stored for 11 days.

	Composition of ethanol-insoluble material	
Constituent	From dressed pears (%)	From peelings (%)
Glucosan (not starch)	21·4	17·6
Starch	2·5	0·2
Galactan	3·5	1·6
Mannan	1·1	3·7
Xylan	21·0	31·0
Araban	10·0	8·4
Polygalacturonic acid	11·5	6·4
Lignin	16·1	23·5
Ash	4·6	1·4
Not accounted for	8·3	6·2

Table 5. *Relative proportions of different polysaccharides in the arbitrary fractions (pectin, hemicelluloses and α-cellulose) extracted from the ethanol-insoluble material of unripe and ripe Conference pears*

Unripe pears were analysed as received from orchard, and ripe pears after storage at +15° for 14 days. Each sample contained 70 pears, mean wt. of each pear 166 g. Six samples were analysed in each group. Range of values given in parentheses.

		Condition of fruit	
Arbitrary fraction	Polysaccharides present	Unripe	Ripe
Pectin	Glucosan*	0·24 (0·12–0·82)	0·35 (0·13–1·3)
	Galactan	0·29 (0·20–0·46)	0·33 (0·26–0·40)
	Xylan	0·08 (0·055–0·12)	0·02 (0·00–0·06)
	Araban	0·78 (0·62–1·05)	1·40 (1·22–1·51)
	Polygalacturonic acid	1·00	1·00
Hemicellulose A	Glucosan	0·20 (0·15–0·24)	0·07 (0·05–0·08)
	Galactan	0·085 (0·05–0·12)	0·04 (0·02–0·05)
	Xylan	1·00	—
	Araban	0·12 (0·08–0·15)	0·02 (0·015–0·03)
	Uronic acid	0·006 (0·004–0·008)	0·001
Hemicellulose B	Glucosan	0·47 (0·30–0·60)	0·21 (0·18–0·26)
	Galactan	0·20 (0·14–0·27)	0·12 (0·10–0·13)
	Mannan	0·07 (0·05–0·10)	0·045 (0·025–0·06)
	Xylan	1·00	1·00
α-Cellulose	Glucosan	1·00	1·00
	Galactan	Trace	Trace
	Mannan	0·14 (0·12–0·15)	0·02 (0·0–0·04)
	Xylan	0·02 (0·0–0·05)	0·12 (0·08–0·14)

* This is soluble glucosan, possibly starch or starch-degradation products. The amount is very variable.

The various fractions (pectin, hemicelluloses and α-cellulose) from six samples (each contained 70 pears, mean weight 166 g.) of ripe and unripe pears were analysed and the results expressed in the form of ratios, the polysaccharide present in largest amount being equated to unity. This method of presentation was used so as to bring out more clearly the variation in the composition of the arbitrary fractions. The six samples of unripe pears were selected from the cases of pears immediately they arrived at the laboratory from the orchard and the six samples of ripe pears after the fruit had been matured for 14 days at +15° (their condition was roughly equivalent to that of the fruit in the main experiment after 11 days, cf. Table 1).

The figures show that the composition of each fraction is very variable, even when samples are at the same stage of maturation, and that the composition of the corresponding fraction changes markedly when unripe pears are compared with ripe. Other experiments have shown that the same is true when pears are compared that have been picked at the same stage of growth and from the same trees in two different seasons (1947 and 1948). Since the composition of the various fractions is not even approximately constant, they cannot be considered as fundamental components of the cell wall.

A much more regular and consistent picture is obtained if the composition of the cell wall is considered in terms of the constituent polysaccharides. The assumption is made that each polysaccharide is built up from only one sugar and that the polysaccharides may appear in a number of fractions. Some results on unripe and ripe pears which have already been partly described in Table 5 are given in terms of the constituent polysaccharides in Table 6.

The glucose produced by the hydrolysis of the water-soluble fraction is assumed to be derived from a different polysaccharide from the glucosan in the holocellulose. Starch is known to be present in the fruit at certain stages of growth, and the soluble glucosan may well be derived from starch.

In the experiments on the effect of ripening on the composition of the pear fruit which are described below, the analytical figures have been expressed in terms of the derived polysaccharides, as in Table 6, and changes in the amounts and composition of the arbitrary fractions pectin, hemicellulose A and B and α-cellulose have been ignored.

Table 6. *Total amount of each polysaccharide in the pear cell wall (minus the soluble glucosan)*

The mean figure, together with the standard error, has been calculated for each polysaccharide from the facts presented in Table 5 (for details of experimental conditions see Table 5).

Polysaccharide	Amount of each polysaccharide (g./pear) Unripe	Ripe
Holocellulose glucosan (cellulose)	0·98±0·065	0·96±0·00
Galactan	0·266±0·03	0·17±0·025
Mannan	0·126±0·008	0·03±0·02
Xylan	0·745±0·06	0·955±0·11
Araban	0·54±0·041	0·48±0·05
Polygalacturonic acid	0·64±0·08	0·34±0·05
Total	3·6±0·20	3·16±0·24

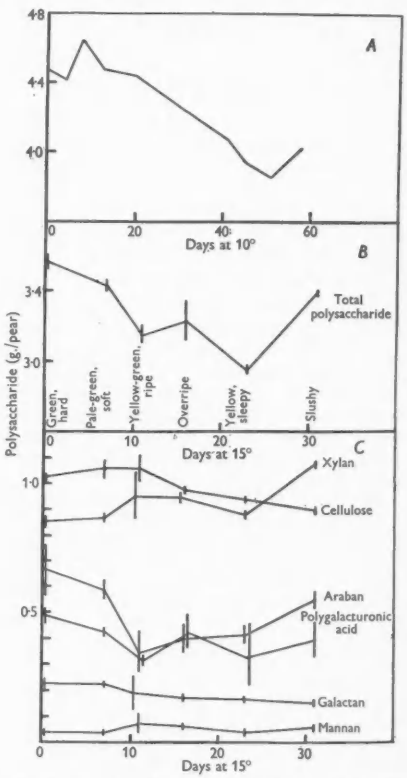

Fig. 1. Changes in the polysaccharide constituents of Conference pears during ripening at +15° and at +10°. The material included polysaccharides which were insoluble in 80% ethanol, such as pectin. *A* represents the changes in the total polysaccharides (including protein, lignin, methoxyl and ash) in pears stored at +10°, and is calculated from the data of Kidd *et al.* (1940). *B* represents the changes in the total polysaccharides in pears stored at +15°, recorded in the present study. *C* represents the changes in the individual polysaccharides corresponding to *B*. *A* has been drawn so as to be roughly comparable with *B* and *C*. The condition of the pears for culinary purposes is indicated by the appropriate description in *B*. The length of the vertical lines indicates the scatter between duplicate estimations.

Table 7. *Changes in the degree of methylation of polygalacturonic acid during storage of pears at +15°*

Figures were calculated from the analyses of the 'pectin' fraction obtained in the main experiment (see text and Tables 1 and 2).

Days of storage	0	7	11	16	23	31
Ester methoxyl (OMe)	2·1	1·5	1·2	0·9	0·5	03·5
(% of cell wall)	1·8	1·9	Nil	0·4	0·5	0·7
Polygalacturonic acid plus OMe as CH_3	11·2	10·2	7·6	11·6	10·3	10·4
(% of cell wall)	10·3	10·6	6·4	8·2	5·4	7·4
Methoxyl content (OMe) of methylated polygalacturonic acid (%)	19·0	15·0	15·7	8·0	4·7	3·4
	17·7	18·3	Nil	4·7	9·6	9·3

Changes in the ethanol-insoluble polysaccharides of pears stored at +15°

The observed changes in the principal polysaccharides present in pear fruit are given in Fig. 1. The range of values between the duplicate estimations are indicated by the extremities of the vertical lines. The value for each point has been corrected for ash, moisture, methoxyl, lignin and nitrogen content in the original fractions and for the loss of sugar during hydrolysis. Most accurately the curves should be described as those for the various anhydro sugars, but it has been assumed, as described previously, that this is equivalent to a first approximation to the corresponding single sugar polysaccharides. In two instances, such polysaccharides, xylan and cellulose, have been isolated in good yield from the appropriate fractions, thus confirming the general correctness of the assumption. The isolation will be described later.

The curves are the mean of two sets of results, which in general follow each other closely. The widest scatter occurs at the stage corresponding to the onset of ripening. This probably means that, at and about the climacteric, such rapid changes are taking place that it is impossible to get samples at exactly equivalent stages of change. There may also be considerable variations in the exact course of the changes through factors which cannot easily be controlled. For example, if an ethylene-producing pear arises at an early stage in the storage of a sample, it will bring on a premature climacteric in adjacent pears and thus anticipate the changes which occur at a later stage in the other samples.

The methoxyl content of the polysaccharides falls during the storage of the pear, and this fall can be accounted for by a fall in the methoxyl content of the pectin fraction. The actual degree of esterification of the polygalacturonic acid in the pectin fraction falls during storage of the fruit. In Table 7 the estimated methoxyl and polygalacturonic acid contents of the pectin fractions are combined to give the methoxyl content of the methylated polygalacturonic acid. Practically all the polygalacturonic acid is extracted in the pectin fraction.

The figures are very variable but indicate that during storage methyl groups are lost from the polygalacturonic acid fraction. Comparison with a fully esterified polygalacturonic acid (methoxyl 16·3 %) indicates that at the start the polygalacturonic acid was probably fully esterified.

Changes in the ethanol-insoluble polysaccharides of pears stored at +5°

Pears stored at lower temperatures ripen more slowly and in some cases develop abnormally. It was of interest, therefore, to examine the changes in the polysaccharide constituents as compared with those in pears ripened at +15°, when both were in approximately the same physical state as judged by colour, firmness and eating quality. The results of an experiment, in which pears stored at +15° for 23 days (sleepy stage of overripeness) were compared with the original unripe pears and with pears stored at +5° for 84 days, are given in Table 8.

The pears at +5° ripened normally and it is noteworthy that the changes in the polysaccharide constituents closely paralleled those at the higher temperature.

Changes in the minor constituents of the cell wall

There are indications of changes in the lignin content during the ripening process which seem to parallel those in the cellulose but, since the estimation of lignin is unreliable and there is considerable variation between duplicates, it is uncertain how far these changes are significant. The observed changes may in fact merely reflect changes in the polysaccharide components which contribute to the production of the insoluble lignin by the method used (72 % H_2SO_4). The figures for lignin, as well as those of ash and nitrogen, have been used to calculate the absolute amounts of polysaccharide in the cell wall, and have not been investigated separately.

Isolation of polysaccharides from main fractions of cell wall

The basic assumption in all the analyses described above has been that, to a first approximation, all the polysaccharides in the cell wall are built up from one

Table 8. *Comparative analysis of the polysaccharides of pears stored at +15° and +5° until they were overripe*

Two samples of pears (50 pears in each, mean wt. of pears 141 g.) were stored in closed containers at +5° for 84 days. They were then in the 'sleepy' stage of overripeness. The figures for the unripe and those ripened at +15° for 23 days are taken from the results of the main experiment (see Tables 1 and 2). Figures are given for each of the duplicates.

	Amount of each polysaccharide (g./pear)					
	Original unripe		After storage at +15° for 23 days		After storage at +5° for 84 days	
Polysaccharide						
Holocellulose glucosan (cellulose)	1·03	1·03	0·92	0·95	0·75	0·75
Galactan	0·24	0·21	0·17	0·16	0·1	0·085
Mannan	0·05	0·03	0·03	0·05	0·03	0·03
Xylan	0·84	0·87	0·87	0·88	0·72	0·75
Araban	0·58	0·76	0·37	0·45	0·25	0·24
Polygalacturonic acid	0·51	0·46	0·45	0·22	0·31	0·37
Soluble glucosan	0·19	0·27	0·13	0·13	0·06	0·04

sugar residue only. Some of the polysaccharides such as polygalacturonic acid, araban and galactan have been isolated from similar fractions of other plant tissues and shown to be of this type, so that it can be assumed with a reasonable degree of certainty that the same is true of the pear. The composition of the hemicellulose and α-cellulose fractions is not so clear, and in the present study these have been examined in detail. From the hemicellulose fractions a pure xylan has been prepared as follows, in good yield calculated on the amount of xylose in the hemicellulose fraction.

Hemicellulose A and B (70 g., extracted from holocellulose with 4N-KOH, as described under Analysis) was dissolved in 4N-KOH and to this solution was added 3·5 l. of a solution (a modified Benedict's reagent) containing 162 g. of potassium citrate, 130 g. of K_2CO_3, and 17·3 g. of $CuSO_4,5H_2O$ per litre. The flask was stoppered, shaken vigorously to coagulate the precipitate and allowed to stand overnight. The deep-blue xylan–copper complex was centrifuged and washed once with 5 l. of N-KOH. The complex was decomposed by stirring into 4 l. of acetic acid. No trace of the blue colour of the complex remained. The mixture was centrifuged and the precipitate of xylan washed on a sintered-glass funnel with 80% (v/v) acetic acid until all the copper had been removed (about 1·5 l. was required). The xylan was washed successively with glacial acetic acid, acetone and ether. Yield, 38 g., 71% of the analytically determined xylan content of the hemicellulose fractions. Hydrolysis with 0·5N-HNO_3 gave xylose 90%, glucose 7%, galactose 3% and a trace of arabinose. To 30 g. of this crude xylan dissolved in 1 l. of N-KOH was added 1 l. of the Benedict's reagent described above, and the mixture was allowed to stand for 2 hr. It was centrifuged, washed on the centrifuge with 2 l. of N-KOH and the precipitate added to 2·5 l. of glacial acetic acid. The precipitated xylan was washed free of copper with 80% acetic acid, then treated with acetone and ether, and air-dried. Yield, 24 g. Hydrolysis showed that not more than 1% of sugars other than xylose were present. A repetition of the purification procedure gave a pure xylan, ash by incineration 0·4%, $[\alpha]_D^{20} - 76°$ in N-NaOH (*c*, 1), Cu 0·018%. Hydrolysis with 0·5N-HNO_3 gave crystalline D-xylose in 91% yield (Heuser & Jayme, 1923), and from the mother liquor was isolated (as crystalline xylose dibenzylidenedimethylacetal) a further 4% (Breddy & Jones, 1945). Total yield, 95%.

The isolation of a degraded cellulose from the α-cellulose obtained from pear cell wall has already been described (Hirst, Isherwood, Jermyn & Jones, 1949). From 30 g. of the α-cellulose, 7·5 g. of the degraded cellulose was obtained. A similar yield of a degraded cellulose was obtained when cotton cellulose was used as starting material, and it appears that the glucose residues in the α-cellulose fraction of pear cell wall are present in the form of the polyglucosan cellulose. The properties of the degraded cellulose from pear cell wall were identical with those of a degraded cellulose obtained in the same way from genuine cotton cellulose.

DISCUSSION

Before the results obtained on pear fruit that have been picked and held in storage are analysed, it is important to know what has happened to the cell wall in the immediate period before the fruit is picked. With the Kieffer pear, Smith (1935) found that the amount of cell-wall material changed very little after a certain point in the natural growth on the tree, and that the increase in size of the fruit was mainly due to water and sugars. In the present study it has been found that the same appears to be true of Conference pears. In Table 9 some figures are given for the cell-wall material of Conference pears picked in different seasons and at different stages of maturity.

The figures indicate that the mean weight of the cell-wall material per pear is much the same whether the fruit are picked early (60 g. in weight) or late (161 g. in weight), and suggest that the cell wall at this time is largely static in nature. This, however, may be a reflexion of a balance between the rate of breakdown of carbon compounds due to respiration and the supply of nutrients from the tree. After picking, the respiration can occur only at the expense of the sources of energy present initially in the fruit, and any balance which may

Table 9. *Absolute weight of cell-wall material per pear at different periods of growth of Conference pears*

The figures are averages from samples of at least 50 pears and include lignin, protein and ash, but not starch.

Source of data	Date of picking	Mean wt. of one pear (g.)	'Alcohol-insoluble content less starch' (%)	Mean wt. of cell-wall material per pear (g.)
Kidd *et al.* (1940)	4. viii. 36	60·2	7·85	4·72
	25. viii. 36	82·6	5·40	4·46
	16. ix. 36	117·5	3·81	4·56
Present study	21. ix. 48	161·5	2·99	4·82
		159·2	2·90	4·62
	20. x. 47	154·0	2·68	4·13

exist in the fruit on the tree is completely changed. If the cell wall is linked with the metabolism of the fruit then this change will affect the composition of the cell wall progressively with time.

Examination of the results shown in Fig. 1 (including those of Kidd *et al.* 1940) indicates that the cell wall is not static but both loses and gains in amount during storage.

The fall in the total polysaccharides is interpreted to mean that at least a part of the carbon compounds locked up in the cell wall became available for conversion into carbon dioxide. The extent to which this occurs depends upon the temperature of storage. Overripeness and tissue breakdown occur more rapidly at higher temperatures and probably set a limit to the orderly breakdown of the cell wall. At $+5°$ the depletion of the cell wall proceeds further than at $+15°$, though at a slower rate. The labile material appears to be mainly araban. The low-temperature storage experiment in particular (Table 8) shows a large loss of this polysaccharide, accompanied by significant losses in galactan and holocellulose glucosan. The decrease in the amounts of these polysaccharides during storage suggests very strongly that the cell wall can act as a reserve substance for the respiration of the cell; the sharp rise at the stage of overripeness indicates that the relationship between the cell wall and the cytoplasm involves synthesis of polysaccharides as well as breakdown. In fact, the cell wall appears to be in dynamic equilibrium with the cytoplasm, and the apparently static nature of the cell wall in the fruit on the tree does not reflect its full relationship with the cytoplasm.

The actual changes that occur in the polysaccharide constituents are calculated in terms of the whole pear, though they are probably mostly confined to the parenchyma cells. The highly lignified thick-walled stone cells do not appear to alter (by microscopic examination, Sterling, 1954) during the maturation of the fruit, and since they represent over half the alcohol-insoluble material the changes (less the relatively constant amount from the stone cells) in the polysaccharides of the cell wall are even more striking than the above figures indicate. This is in agreement with microscopic examinations which show that when the fruit are overripe the walls of the parenchyma cells become extremely thin and tenuous, and in some areas may separate and collapse.

SUMMARY

1. The cell-wall material of the Conference pear that is insoluble in ethanol was divided into fractions by extraction with boiling water and cold alkali. Each fraction was hydrolysed and the mixture of sugars separated on a paper chromatogram and estimated colorimetrically.

2. It is assumed that each sugar in the hydrolysis mixtures corresponded to a single polysaccharide without regard to the fraction from which it was derived, and the results have been interpreted in terms of the constituent polysaccharides of the cell wall. The changes that occur in the amounts of these single-sugar polysaccharides (xylan, araban, polygalacturonic acid, galactan and cellulose) have been followed during the storage of the pear fruit at $+15°$ and $+5°$.

3. Changes in the amount of the total cell wall and the fractions into which it is conveniently divided, 'pectin', 'hemicellulose', 'cellulose', are a reflexion of the separate changes in the amounts of the individual polysaccharides. The results indicate that the cell wall of the pear appears to be in dynamic equilibrium with the cytoplasm, and polysaccharides are both broken down and synthesized during the physiological changes which take place during ripening.

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The Synthesis of Indole by Washed Cell Suspensions of *Escherichia coli*

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The biosynthesis of the aromatic amino acids by micro-organisms has been mainly investigated by studies on the nutrition of normal and mutant cells and the accumulation of intermediate compounds in culture media. Recently several of the reactions concerned in aromatic synthesis in *Escherichia* and *Aerobacter* have been examined in detail with cell-free extracts (Kalan & Srinivasan, 1955; Mitsuhashi & Davis, 1954; Yaniv & Gilvarg, 1955; Yanofsky, 1955).

During an investigation of the formation of indole by bacteria it was found that washed-cell suspensions of certain mutants of *Aero. aerogenes* and *Esch. coli* would form aromatic compounds from glucose and ammonium salts. The formation of indole, anthranilic acid and an unidentified indole-like compound has been briefly reported by Gibson, Jones & Teltscher, 1955. This paper concerns the formation of indole.

MATERIALS AND METHODS

Strains of Esch. coli *used.* These (Table 1) were mutant strains derived originally from *Esch. coli* 518. They were obtained after ultraviolet irradiation and penicillin selection by the technique described by Davis (1948). Strains were maintained by serial subculture on heart-infusion agar.

Glassware and chemicals. Glassware was cleaned with a chromic acid cleaning mixture. The chemicals were the purest obtainable commercially; no attempt was made to purify them further.

Media. Two media were used for the routine preparation of cell suspensions.

(*a*) *Heart-infusion agar.* To 1 l. of tap water were added 500 g. of minced defatted ox heart, 10 g. of Evans peptone and 5 g. of NaCl. After steaming for 1 hr. the broth was filtered, the pH adjusted to 8·4, and the liquid autoclaved (15 lb./in.^{2} for 20 min.) and re-filtered. Agar (1·5%) was added, and after autoclaving at 15 lb./in.^{2} for 20 min. to dissolve the agar the pH was adjusted to 7·2–7·4. The medium was then autoclaved at 15 lb./in.^{2} for 20 min.

(*b*) *Synthetic medium.* The medium used was that described by Davis & Mingioli (1950). Glucose was added with the other ingredients before autoclaving. Double-strength liquid medium dispensed in 7·5 ml. volumes was sterilized at 10 lb./in.^{2} for 10 min. and added aseptically to an equal volume of sterile molten 3% agar.

Preparation and use of cell suspensions. Agar plates (4 in. diam.) were inoculated by spreading over them 0·2 ml. of a light (approx. 4×10^6 cells/ml.) suspension of organisms in distilled water. The inoculum was taken from a heart-

Table 1. *Strains of* Esch. coli

Mutant no.	Strain	Growth requirement*	Parent strain
—	518	None	—
1	7–4	Tryptophan	518
2	T2–15	Tryptophan (10^{-4} M, DL) plus serine (10^{-3} M, DL) or plus glycine (10^{-3} M)	7–4

* Supplement needed for growth in minimal medium (Davis & Mingioli, 1950), and concentration used during present experiments.

infusion agar slope incubated for 24 hr. at 37°. The inoculated plates were incubated for 16 hr. at 37° and the cells washed off with 0·9% NaCl solution. For cells grown on heart-infusion agar 10 ml. of saline was used per plate, and for synthetic medium agar, 5 ml. of saline. The cells were washed twice with saline and resuspended in 0·2 M phosphate buffer, pH 7·7 (Na_2HPO_4–KH_2PO_4). The density of the suspension was measured turbidimetrically and adjusted to approx. 1·5 mg. dry wt./ml. Freshly harvested cells were used for each experiment.

Tests for indole synthesis were carried out by adding a suspension of organisms prepared as described to an equal volume of the mixture of substrate solutions to give a final volume of 5 ml. in 6 in. × ¾ in. tubes. The tubes were then incubated in a water bath at 37°.

Generally no increase in turbidity was observed in the course of an experiment, indicating that no growth was taking place. In a few experiments some increase in turbidity was observed which, however, did not exceed 50% of the original turbidity. For example, in experiments with mutant 1, when tryptophan and $MgSO_4$ were included in the synthesis system the turbidity increased by about 50%.

Assay of indole. Indole was estimated by a modification of the method of Wood, Gunsalus & Umbreit (1947). To 6 ml. of toluene was added 0·5 ml. of sample. After shaking for 2 min., 3 ml. of the toluene layer was transferred to a fresh tube and 1 ml. of a 5% solution of *p*-dimethylaminobenzaldehyde in ethanol added, followed by 1 ml. of acid alcohol (500 ml. of ethanol plus 40 ml. of conc. H_2SO_4). The colour was allowed to develop for 12–15 min. and the absorption compared with a reagent blank by means of an EEL portable colorimeter with ½ in. tubes and a 626 mμ. filter. A standard curve was prepared with known concentrations of indole. Results are expressed in terms of μm-moles of indole formed/mg. dry weight of cells.

Confirmation of identity of indole. The formation of indole was confirmed by growth tests with mutants responding to indole, and by comparison of the ultraviolet absorption spectra of experimental samples with those of solutions of indole (Gibson *et al.* 1955).

RESULTS

Mutant 1 required tryptophan for growth (Table 1). Since tryptophan cannot be replaced for this organism by indole or anthranilic acid the pathway of tryptophan synthesis is presumably blocked between indole and tryptophan. This has been confirmed by experiments with washed-cell suspensions of mutant 1, which failed to form tryptophan from indole plus serine, although the same suspensions did form indole from tryptophan.

'Cross-feeding' experiments with the technique described by Gibson & Jones (1954) indicated that mutant 1 accumulated indole in culture, supporting the growth of strains requiring indole.

Washed-cell suspensions of mutant 1 formed indole from glucose and ammonium chloride. Examination of a number of other strains in which tryptophan synthesis was apparently blocked at the same stage as that of mutant 1 showed that there was a constant correlation between the ability of a strain to 'cross-feed' indole-requiring strains in culture and the ability of washed-cell suspensions to form indole.

The amount of indole formed by suspensions of mutant 1 varied greatly when the cells were harvested from different media. The experiments to be described have therefore been grouped according to the media from which the test cells were harvested.

Indole synthesis by cells grown on heart-infusion agar

When suspensions of mutant 1, harvested from heart-infusion agar, were incubated with glucose and ammonium chloride, indole was formed in considerable amounts (Table 2). Ammonium chloride could be replaced by other ammonium salts such as ammonium nitrate or ammonium sulphate. The optimum concentration of glucose was 0·1 M, and that of ammonium chloride 0·005 M. Preliminary experiments showed that with 0·1 M phosphate buffers the optimum initial pH for indole synthesis lies in the range 7·5–8·0. There was a considerable drop in pH during the course of the experiments, for example from pH 7·7 to 6·5–7·0.

Many amino acids when added to the glucose–ammonium chloride medium had no significant effect upon indole synthesis by mutant 1, but several inhibited the synthesis and one, serine, stimulated it (Tables 2 and 3).

Serine therefore might be essential for some reaction on the pathway of indole synthesis, and, if so, mutant 1 must synthesize some serine from glucose and ammonium chloride. It appeared that by using a strain of *Esch. coli* which could not form serine from these substrates it would be possible to control the amount of serine available to the cells. Irradiation of mutant 1, followed by penicillin selection, gave mutant 2, which required for growth, in addition to tryptophan, either serine or glycine

Table 2. *Effect of serine on indole synthesis by mutant* 1

Suspensions were incubated for 4 hr. at 37° with substrates in 0·1 M phosphate buffer, pH 7·7. In this and all further experiments shown, the amount of cells present was within the range 0·64–0·80 mg. dry wt./ml. $MgSO_4$ (10^{-4} M) was added to substrates for cells harvested from synthetic medium. +, Compound was added to suspension; −, compound was not added.

Substrate	Cells harvested from Heart-infusion agar			Synthetic medium		
Glucose (0·1 M)	+	+	+	+	+	+
NH_4Cl (0·005 M)	−	+	+	−	+	+
DL-Serine (0·01 M)	−	−	+	−	−	+
Indole formed (μm-moles/mg. dry wt.)	39	188	286	<10	238	325

(Table 1). Mutant 2 presumably could not form serine except from glycine.

In contrast with mutant 1, mutant 2 did not form any more indole from glucose and ammonium chloride than from glucose alone (Fig. 1). When serine was added indole was formed in considerable amount. DL-Serine was used in the experiments described and D-serine was found to be inactive.

Table 3. *Effect of amino acids on indole formation by* Esch. coli 7–4

Amino acids added to give 0·01 M final concentration to glucose (0·1 M) and NH_4Cl (0·005 M) in 0·1 M phosphate buffer, pH 7·7. Summary of several experiments. Incubation time, 3–5 hr. at 37°.

Amino acid	Effect
DL-Serine	Variable stimulation (15–100%)
DL-Alanine, L-arginine, DL-aspartic acid, DL-histidine, L-hydroxyproline, DL-isoleucine, DL-leucine, DL-methionine, DL-proline, DL-tyrosine, DL-valine	No stimulation or inhibition less than 10%
L-Cysteine, DL-glutamic acid, glycine, DL-homoserine, DL-lysine, DL-phenylalanine, DL-threonine, DL-tryptophan	Inhibition, greater than 10%

No other amino acids would completely replace serine for indole synthesis by mutant 2, and the only others which were at all effective were glycine, threonine and homoserine, in that order. The amounts of indole formed in the presence of serine and the three amino acids mentioned above are shown in Fig. 1. Under the conditions shown in Fig. 1 the omission of ammonium chloride did not affect indole formation when DL-serine was present. Serine was rapidly deaminated and could therefore act as a source of ammonium ions. For optimum synthesis with glycine as substrate, ammonium ions were essential and they were therefore included in all experiments. The effects of the four amino acids have been examined in greater detail with cells harvested from synthetic medium and are described below.

In Fig. 2 the amount of indole formed by mutant 1 with serine present is plotted against time. The curve shows an initial lag phase followed by a period in which indole is formed at a constant rate. The length of the lag period and the constant rate of formation varied somewhat in different experiments, but were of the order of 2 hr. and 100 μm-moles of indole formed/mg. dry weight of cells/hr., respectively.

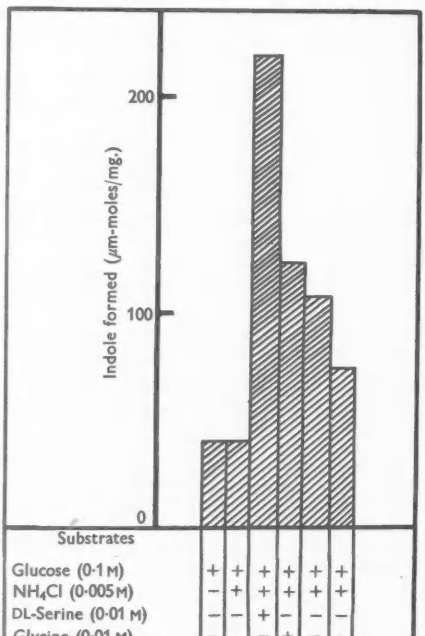
Indole formed (μm-moles/mg.)
200
100
0
Substrates
Glucose (0·1 M) + + + + + +
NH4Cl (0·005 M) − + + + + +
DL-Serine (0·01 M) − − + − − −
Glycine (0·01 M) − − − + − −
DL-Threonine (0·01 M) − − − − + −
DL-Homoserine (0·01 M) − − − − − +

Fig. 1. Effect of serine, glycine, threonine and homoserine on indole synthesis by cells of mutant 2 grown on heart-infusion agar. Cells were incubated with substrates as shown, in 0·1 M phosphate buffer, pH 7·7, for 5 hr. at 37°.

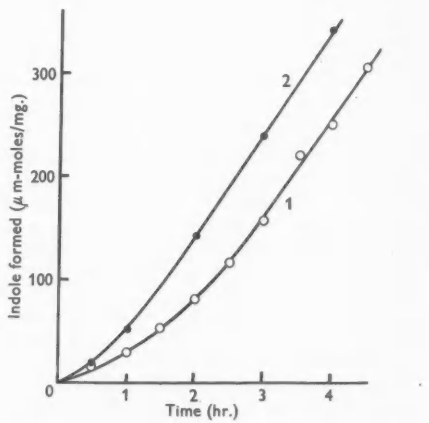

Fig. 2. Rate of indole synthesis by mutant 1. Curve 1: cells from heart-infusion agar incubated at 37° in 0·1 M phosphate buffer, pH 7·7, with glucose (0·1 M), NH_4Cl (0·005 M) and DL-serine (0·01 M). Final vol., 17 ml. Curve 2: cells from synthetic agar incubated as above with the further addition of $MgSO_4$ (10^{-4} M). Final vol., 10 ml.

Indole synthesis by cells grown on synthetic medium

Cell suspensions of mutant 1 harvested from synthetic medium containing tryptophan (10^{-4} M) formed less indole in a given time than did suspensions harvested from heart-infusion agar. Indole synthesis was stimulated by the addition of cell extracts, obtained as the supernatants after treating heavy cell suspensions of mutant or wild-type *Esch. coli* with boiling water and centrifuging. Various mixtures of vitamins or amino acids failed to replace the cell extracts, which could, however, be replaced by magnesium salts. A number of salts were tested to see if they would also stimulate indole formation. From the results shown in Table 4 it can be seen that, at the concentrations tested, magnesium and manganous ions both stimulated indole synthesis, and zinc and cobalt caused inhibition. At a concentration of 10^{-5} M, magnesium stimulated synthesis much more effectively than any other metal. In some experiments Fe^{2+} ions stimulated indole formation. The concentrations of Mg^{2+} ions needed to stimulate synthesis are shown in Fig. 3.

As with cells grown on heart-infusion agar, indole formation by mutant 1 was stimulated by serine (Table 2) and there was a lag period before a constant rate of synthesis was reached (Fig. 2).

For the growth of mutant 2 either serine or glycine, in addition to tryptophan, had to be added to the synthetic medium (Table 1). Cells harvested from either a glycine or a serine medium failed to form indole from glucose and ammonium chloride, but would do so if serine was added. Other amino acids were tested to see if they would replace serine, and again only glycine, threonine or homoserine would promote indole formation.

Table 4. *Effect of metals on indole synthesis by mutant* 1 *harvested from synthetic medium*

Salts as shown were added to indole synthesis system consisting of cells, glucose (0·1 M), NH_4Cl (0·005 M) and DL-serine (0·01 M) in 0·1 M phosphate buffer, pH 7·7. Incubation time, 4 hr. at 37°. In the control no salt was added and the indole formed was 133 μm-moles/mg.

Salt added	Indole formed (μm-moles/mg.) in the presence of salt added to final concn. of 10^{-5} M	10^{-4} M
$CaSO_4$	117	121
$AlK(SO_4)_2$	117	111
Na_2SO_4	117	122
$ZnSO_4$	122	10
$CoSO_4$	42	9
$Fe_2(SO_4)_3$	138	132
$FeSO_4$	142	144
$MnSO_4$	136	248
$MgSO_4$	310	316

The amounts of indole formed with different amino acids as substrates were markedly influenced by the medium on which the cells were grown. As can be seen from Fig. 4, with cells harvested from a glycine medium, the effectiveness of serine, glycine, threonine and homoserine as substrates for indole synthesis diminished in that order. With cells grown on a serine medium, serine was most effective in promoting indole formation. Glycine, threonine and homoserine gave rise to only a small amount of indole (Fig. 4).

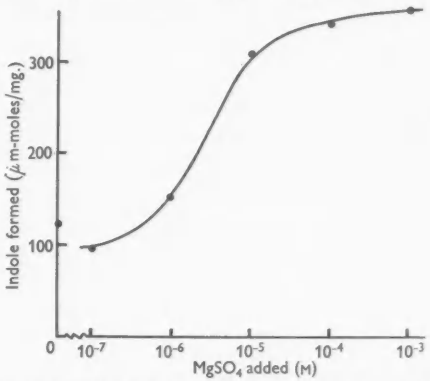

Fig. 3. Stimulation of indole synthesis by Mg^{2+} ions. Cells from synthetic medium agar incubated at 37° with glucose (0·1 M), DL-serine (0·01 M), NH_4Cl (0·005 M) and $MgSO_4$ as shown, for 4 hr. in 0·1 M phosphate buffer, pH 7·7.

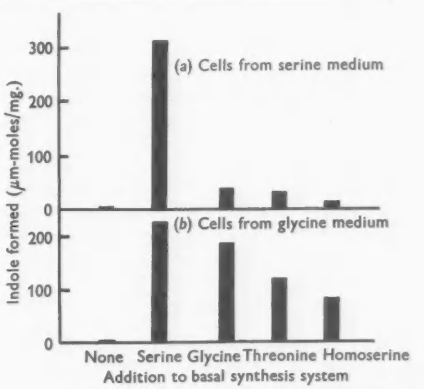

Fig. 4. Serine, glycine, threonine and homoserine as substrates for indole synthesis: mutant 2 grown on synthetic media containing (*a*) serine or (*b*) glycine. Basal synthesis system; glucose (0·1 M), NH_4Cl, (0·005 M) and $MgSO_4$ (10^{-4} M). Cells incubated at 37° in 0·1 M phosphate buffer, pH 7·7. Incubation time (*a*) 5 hr., (*b*) 4 hr. Concentration of amino acid, 0·01 M.

Table 5. *Effect of growth medium and magnesium on indole formation*

Cells were incubated in 0·1 M phosphate buffer, pH 7·7, for 4 hr. at 37° with substrates shown.

	Indole formed in	
Agar growth medium	Basal system* (μm-moles/ mg. dry wt.)	Basal system* + $MgSO_4$ (10^{-4} M) (μm-moles/ mg. dry wt.)
Heart-infusion	286	336
Heart-infusion + 10^{-3} M DL-tryptophan	63	237
Difco heart-infusion	63	246
Difco proteose no. 3	29	382
Difco tryptose	23	248
Difco brain–heart-infusion	152	242
Difco yeast–beef	24	133

* Basal system: glucose, 0·1 M; NH_4Cl, 0·005 M; DL-serine, 0·01 M.

Indole synthesis by cells grown in various other complex media

Early attempts to replace the stock laboratory heart-infusion agar with Difco heart-infusion agar or other Difco media yielded suspensions of cells which formed relatively little indole. This is shown in the results of the experiments summarized in Table 5 (middle column).

It was thought, for reasons which are given below, that the concentration of tryptophan in the growth medium might influence indole formation. Cells harvested from heart-infusion agar to which had been added 10^{-3} M DL-tryptophan did form much less indole than cells grown on heart-infusion agar alone, as can be seen from the results shown in Table 5 (middle column).

After the importance of magnesium in indole formation had been established the problem of relatively 'inactive' cells was reinvestigated. It was found (Table 5) that synthesis by 'inactive' cell suspensions, whether they were obtained by growth on Difco media or by the addition of tryptophan or indole to heart-infusion agar, was greatly stimulated by magnesium.

DISCUSSION

The results presented show that indole is formed from glucose and ammonium salts by washed-cell suspensions of a mutant strain of *Esch. coli* (mutant 1) unable to convert indole into tryptophan. The fact that cell suspensions will form indole from these simple precursors indicates that their use may be of value in the study of aromatic synthesis.

Serine, or a product of serine metabolism other than glycine, is probably concerned with some stage in indole synthesis. Several experimental findings support this suggestion. First, serine alone of the amino acids tested stimulated indole formation by mutant 1. Secondly, with another mutant (2) isolated from mutant 1 it was possible to limit the amount of serine available to the indole-synthesis system. Suspensions of mutant 2 would not form indole from glucose plus ammonium chloride but required the addition of serine or a related amino acid. If the cells were grown on a synthetic medium containing serine, only serine was effective in promoting active indole formation. Cells harvested from a glycine medium would use in decreasing order of effectiveness serine, glycine, threonine and homoserine as substrates for indole synthesis. As suspensions of cells of *Esch. coli* requiring serine or glycine for growth readily form glycine from homoserine or threonine (Gibson & Woods, unpublished observations) the most likely explanation of the activity of the latter two amino acids is that they are converted through glycine into serine, which is the intermediate most closely concerned with indole formation.

The stage at which serine acts as an intermediate in indole formation is unknown, although preliminary experiments on anthranilic acid formation by washed-cell suspensions (Gibson *et al.* 1955) indicate that it is probably before the anthranilic acid stage.

Although no attempts were made during the experiments to prepare cells deficient in metal, the addition of some metals, notably magnesium, was found to stimulate indole synthesis under certain conditions. Knowledge of the stage or stages in indole synthesis at which these metals function must await further work on the individual reactions concerned in the metabolic pathway.

Since cells grown on a glycine synthetic medium were more efficient at using glycine and related amino acids as substrates for indole synthesis than cells grown on a serine medium, it is probable that there is some adaptive process concerned in the conversion of glycine into serine. Similar results have been found with regard to the utilization of serine and glycine as substrates for the methylation of homocysteine (Gibson & Woods, unpublished observations). In addition, the initial increasing rate of indole formation suggests an adaptive process.

One of the reasons for the differing activities of the cell suspensions of mutant 1 harvested from various complex media may be the tryptophan concentration in the medium. Thus the addition of tryptophan to the stock heart-infusion agar gave cells of low activity. It was thought originally that mutant 1 may have produced indole from the tryptophan in the medium, which in turn depressed indole synthesis in a manner similar to that described for

methionine and the synthesis of methionine by cell suspensions of *Esch. coli* (Wijesundera & Woods, 1953; Cohn, Cohen & Monod, 1953). However, the addition of indole to the growth medium stimulated rather than depressed indole synthesis, therefore the effect of tryptophan remains unexplained.

SUMMARY

1. Indole is formed from glucose and ammonium salts by washed-cell suspensions of a mutant strain of *Escherichia coli* (strain 7–4) unable to convert indole into tryptophan.

2. The medium on which the cells are grown influences the amount of indole formed by the harvested cells.

3. Magnesium ions greatly stimulate indole formation by cells grown on either a synthetic medium or on various complex media. Manganous and ferrous ions stimulate to a lesser extent.

4. Serine appears to be concerned in indole formation. This is shown by experiments with cell suspensions of both *Esch. coli* 7–4 and a further mutant (*Esch. coli* T2–15) which requires tryptophan plus serine or glycine for growth.

We wish to thank Miss Barbara McDougall, B.Sc., for her assistance with some of the experiments, Dr B. D. Davis for cultures of *Aero. aerogenes* mutants which were used in preliminary experiments, and Professor S. D. Rubbo for his encouragement of this work. The work was supported in part by a grant from the National Health and Medical Research Council.

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Synthesis and Metabolism of some Substances Related to δ-Aminolaevulic Acid

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In a preliminary communication (Neuberger & Scott, 1953) reasons were given which had led us to believe that δ-succinamidolaevulic acid might be an intermediate in the biosynthesis of porphyrins. But this succinamido compound, unlike δ-aminolaevulic acid (ALA) itself, was not converted into porphyrins by the intact cells of *Rhodopseudomonas spheroides*, as observed by Dr J. Lascelles, nor did it show any activity, according to the experiments of Drs E. Dresel and J. E. Falk, in their system of lysed fowl red cells. ALA itself is the immediate precursor of porphobilinogen (PBG), which in turn gives rise to porphyrins (Shemin & Russell, 1953; Neuberger & Scott, 1953; Dresel & Falk, 1953). In the present paper further observations with rats are presented which indicate that succinamidolaevulic acid is not converted into ALA to a significant extent. Experiments are also reported which indicate that *N*-succinylglycine and *N*-β-carboxypropionylglycine, unlike *N*-acetylglycine or *N*-propionylglycine, are not converted into glycine in the rat. This suggested that the negative results obtained with succinamidolaevulic acid might be due to a general inability of the organism to split the succinamido bond. δ-Acetamidolaevulic acid was therefore synthesized, but biological tests indicate that this compound is not hydrolysed in the body to a significant extent.

It is now assumed that the condensation of glycine and 'active' succinate gives rise in the first place to α-amino-β-oxoadipate, which loses one of its carboxyl groups to yield ALA. Free β-keto acids containing an α-amino group have so far not been prepared, and nothing is known of the stability of compounds of this type. As a preliminary to a possible synthesis of the α-amino-β-keto acid itself, several esters of the acid have been synthesized, and

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the behaviour of these substances in different biological systems has been compared with that of ALA.

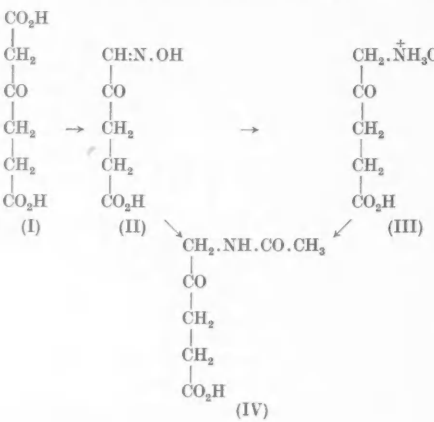
Synthesis of compounds

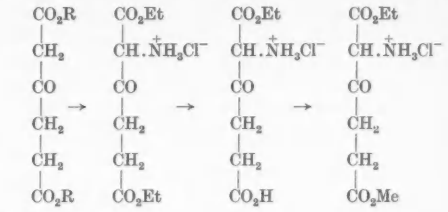
The synthesis of δ-aminolaevulic acid by two routes, and that of δ-succinamidolaevulic acid, of δ-succinimidolaevulic acid and of *N*-β-carboxypropionylglycine, have already been described (Neuberger & Scott, 1954). Independently, Shemin, Russell & Abramsky (1955, see also Shemin & Russell, 1953) have synthesized aminolaevulic acid by three methods, one starting from β-oxoadipic acid, the second with a Gabriel condensation similar to that used in one of our two methods, and the third consisting of a Bamberger fission of β-imidazolylpropionic acid. Succinylglycine was prepared according to Scheiber & Reckleben (1913).

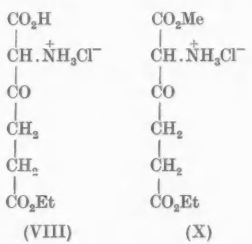
δ-Acetamidolaevulic acid was synthesized in two ways: β-oxoadipic acid (I; Eisner, Elvidge & Linstead, 1950) was converted by treatment with $NaNO_2$ into δ-oximinolaevulic acid (II); the latter was reduced by catalytic hydrogenation to δ-aminolaevulic acid (III) or by reductive acetylation to δ-acetamidolaevulic acid (IV). However, the yield in the latter step was not good, and direct acetylation of (III) with acetic anhydride in acetic acid was more satisfactory.

In order to acquire more knowledge of the chemistry of α-amino-β-oxoadipic acid a number of its esters were synthesized, and their hydrolysis has been studied under a variety of conditions. The diethyl ester has also been obtained by Shemin & Weliky (unpublished results quoted by Shemin *et al.* 1955),

Ethyl β-oxoadipate (V*a*), which is most conveniently prepared from ethyl α-carbethoxy-β-oxoadipate by the method of MacDonald & Stedman (1955), was converted by treatment with ethyl nitrite into the α-oximino derivative, and the latter



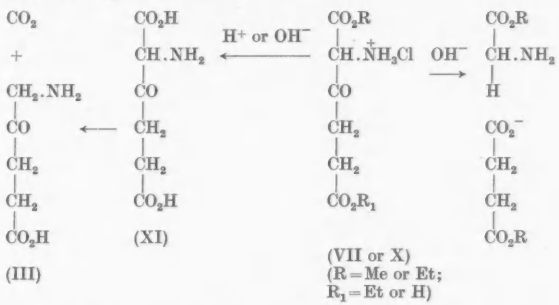




was catalytically reduced to the amino acid ester (VI) following the direction of Harington & Randall (1931), who had used the next lower homologue. The hydrochloride of the diester (VI) had m.p. 155–156° and was readily converted by boiling with concentrated HCl into δ-aminolaevulic acid hydrochloride (III). Treatment with acid under milder conditions gave in good yield a monoethyl ester. This half-ester is assumed to have the structure (VII) rather than (VIII) for the following reasons: (*a*) in the partial hydrolysis of (VI) the approach of the catalytically active proton to the δ-ester group will be less hindered by Coulomb forces than that to the α-ester group, which is adjacent to the positively charged ammonium group. It might thus be expected that, if the possible effect of the β-keto group is disregarded, the δ-ester group should be hydrolysed by acid at a greater rate than the α-ester group. (*b*) The half-ester was found to be stable up to a temperature of about 140°. This is not surprising for a compound of structure (VII), but (VIII), being a free β-keto acid, would be expected to be decarboxylated fairly readily. (*c*) The pK_1 of the half-ester was found to be 4·05; the magnitude of this pK which corresponds with the ionization of the carboxyl group is similar to that of the pK_1 of α-ethyl hydrogen glutamate (3·84; Neuberger, 1936). If the half-ester had the structure (VIII), a pK_1 value of about 1·8–2·1, slightly lower than that of γ-ethyl hydrogen glutamate (2·15), would have been expected. The half-ester (VII), on treatment with ethanolic HCl, gave the diethyl ester (VI), and on treatment with methanolic HCl under mild conditions gave the ethyl methyl diester, which must have structure (IX) (isomer A).

An ethyl methyl diester of α-amino-β-oxoadipic acid that was not identical with (IX) was unintentionally obtained in the attempted synthesis of the dimethyl ester. Methyl β-oxoadipate (V*b*), made by treatment of (I) with diazomethane, was allowed to react with ethyl nitrite, and the oximino derivative was reduced to an amino acid ester as described above. Analysis showed that the product was not, as expected, a dimethyl ester, but apparently an ethyl methyl ester. The compound was a derivative of α-amino-β-oxoadipic acid, since it yielded (III) or glycine on hydrolysis under suitable conditions. The m.p. of this diester hydrochloride was similar to that of (IX), but the mixed m.p. was greatly depressed. Moreover, whilst the new diester (isomer *B*, X) gave readily a crystalline *N*-benzyloxycarbonyl derivative (see below), the isomer *A* (IX) gave only an oily product, which could not be induced to crystallize by seeding with the crystalline derivative of (X). It thus appeared that the new diester must have structure (X). Transesterification must have occurred either during the reduction of the oximino compound, which was done in ethanolic–aqueous HCl, or during the recrystallization of the amino acid ester hydrochloride from ethanol–ether. The preferential transesterification which occurred in the ω-position must be due to the electrostatic factors already discussed.

Heating the diester (X) with 5N-HCl at 100° for 1 hr. gave only δ-aminolaevulic acid hydrochloride, with evolution of carbon dioxide. Treatment of the diester at room temperature with 2N-HCl and 7N-HCl for different periods gave mixtures of crystalline substances which, on paper-chromatographic analysis, were shown to contain varied proportions of starting material, a half-ester (probably the α-methyl ester) and aminolaevulic acid. Alkaline hydrolysis under a variety of conditions produced mixtures of glycine and aminolaevulic acid, the proportion of the former increasing with alkalinity. There was no evidence that significant amounts of the desired α-amino-β-keto acid accumulated in any of these experiments. It would appear that with acid the ester groups are split to give α-amino-β-oxoadipic acid (XI) but that the latter readily loses carbon dioxide under the conditions required for the hydrolysis. In alkaline solution there is, in addition to the ester hydrolysis and decarboxylation, an attack of the hydroxyl ion on the ester (X) which results in a C_2–C_3 fission, producing in the first place glycine ester and ethyl hydrogen succinate. Further attack by hydroxyl ions then yields the free acids. Similar results were obtained with the half-ester (VII).



It appeared possible that the ease with which (XI) loses carbon dioxide in acid solution might be due to the presence of the α-amino group, which carries a positive charge in an acidic or neutral medium. *N*-Benzyloxycarbonyl derivatives of the esters (VII) and (X) were therefore prepared, and it was hoped that these might be converted into the free acyl derivative of (XI) under mild conditions. Catalytic reduction would then yield the desired compound (XI). However, treatment of these acyl derivatives in aqueous ethanol with 2 or 3 equivalents of alkali at room temperature yielded only unchanged starting material. The use of larger quantities of alkali at various temperatures gave a compound melting at 103–104°, afterwards identified as δ-benzyloxycarbamidolaevulic acid, and *N*-benzyloxycarbonylglycine. The former was identified by comparison with a specimen synthesized from δ-aminolaevulic acid and *N*-benzyloxycarbonyl chloride. This acylamidolaevulic acid, after melting at 103–104°, was found to solidify and to remelt at 124–125°. It was thought that this might be caused by a conversion of the acid into either an enolic lactone, i.e. a butenolide, or a hydroxylactone. The former possibility was excluded, since elementary analysis indicated that the conversion was not associated with a loss of water. The second possibility is unlikely, since solution of both materials in aqueous ethanol gives the same pH. Moreover, dissolving material of m.p. 124–125° in alkali, followed by acidification, yielded a specimen which after air-drying had unchanged m.p. It thus appears that δ-benzyloxycarbamidolaevulic acid

occurs in two crystalline modifications. The lower-melting material is the more unstable, since our recent preparations have yielded directly the higher-melting form.

EXPERIMENTAL

Synthesis of compounds

Melting points are uncorrected. Paper chromatography was done throughout with Whatman no. 1 paper and *n*-butanol–water–acetic acid (63:27:10, by vol.).

δ-Oximinolaevulic acid (II). To a solution of *β*-oxoadipic acid (0·82 g.) in 10 ml. of water was added at 0° 2N-NaOH (4·75 ml., 1·95 equiv.), followed by $NaNO_2$ (0·3 g. in 3 ml. of water). To the mixture after standing for 0·5 hr. at 0° was added slowly with shaking 2N-H_2SO_4 (7·5 ml.). The solution was then allowed to warm up to 20° and concentrated *in vacuo* to one-third of its volume. A precipitate which formed on addition of ethanol (60 ml.) was filtered off and the solution was concentrated *in vacuo* to dryness. The residue was extracted with several portions of anhydrous ether. The combined ether extracts were concentrated to dryness and the residue was recrystallized first from anhydrous ether and then from chloroform. *δ-Oximinolaevulic acid* had m.p. 115–116°. The yield was 35% of the theoretical. (Found: C, 41·3; H, 5·1; N, 9·0, 8·9, 9·1. $C_5H_7O_4N$ requires C, 41·4; H, 4·8; N, 9·6%.)

A solution of the oximino acid (0·97 g.) in 15 ml. of N-HCl containing palladium–charcoal (0·5 g.) was shaken in an atmosphere of hydrogen. The theoretical amount of gas was taken up in 25 min. The solution remaining after removal of the catalyst was concentrated *in vacuo*. The residue was crystallized from dry ethanol–ether and from dry ethanol–ethyl acetate. The compound had m.p. 142–143°; the mixed m.p. with an authentic sample of δ-aminolaevulic acid hydrochloride (m.p. 145°) was 143–144°. The R_F value of the compound was 0·23, which is identical with that reported for aminolaevulic acid (Berlin, Neuberger & Scott, 1956).

Catalytic reduction of the oximino acid with palladium–charcoal in glacial acetic acid containing acetic anhydride was sluggish and incomplete after 5 hr. A small amount of material of m.p. 97° was obtained, which was later shown to be δ-acetamidolaevulic acid. The yield was only 5%. It was found more convenient to prepare this compound by acetylation of aminolaevulic acid.

δ-Acetamidolaevulic acid. To a solution of δ-aminolaevulic acid hydrochloride (0·5 g.) in acetic acid (10 ml.) were added successively acetic anhydride (0·3 ml.) and anhydrous sodium acetate (246 mg.). The mixture was shaken for 0·5 hr. and then allowed to stand for 18 hr. A precipitate was removed by centrifuging and the solution was concentrated *in vacuo* to dryness. The residue was dissolved in ethanol (10 ml.) and anhydrous ether (10 ml.) was added. The resulting precipitate was discarded. The ethanol–ether solution was concentrated to dryness and the residue was dissolved in a very small volume of hot ethanol. On addition of ether a crystalline precipitate appeared which was recrystallized from ethyl acetate. The *δ-acetamidolaevulic acid* had m.p. 97°. Yield was 55%. (Found: C, 48·5; H, 6·6; N, 7·9. $C_7H_{11}O_4N$ requires C, 48·5; H, 6·4; N, 8·1%.)

δ-Benzyloxycarbamidolaevulic acid. To a solution of δ-aminolaevulic acid hydrochloride (0·335 g.) in water (7 ml.), cooled to about 5° and containing MgO (0·2 g.), was added in portions and with intermittent shaking benzyloxycarbonyl chloride (0·4 ml.) over a period of 20 min. The mixture was then allowed to stand at room temperature for 1 hr. and filtered. The filtrate was extracted with ethyl acetate and acidified. The precipitate which formed was filtered off and dried. On recrystallization from ethyl acetate–light petroleum (b.p. 60–80°) *δ-benzyloxycarbamidolaevulic acid* was obtained. The yield was 55%. [Found: C, 58·6; H, 5·5; N (Kjeldahl), 5·4; $C_{13}H_{15}O_5N$ requires C, 58·9; H, 5·7; N, 5·3%.] The substance had m.p. 103–104°, resolidified at about 110° and remelted at 124–125°. Material of m.p. 103–104°, which was heated *in vacuo* at 80° in the presence of P_2O_5 for 2 hr., did not melt at 103–104°, but had m.p. 125°. The analysis of the heated material showed that no change in elementary composition had been produced. (Found: C, 58·7; H, 5·5%.) Samples of materials having m.p. 103–104° and 125° respectively were titrated electrometrically in 50% (by vol.) ethanol with a glass electrode. The apparent initial pH in both cases was 3·7; both materials gave normal titration curves on addition of 0·1N-NaOH, the end-points corresponding to equivalent weights of 263 and 268 respectively. ($C_{13}H_{15}O_5N$ requires equiv. wt. 265.) Material of m.p. 125° (50 mg.) was dissolved in a slight excess of aqueous alkali. Acidification gave a crystalline precipitate which was filtered off and air-dried; m.p. was unchanged. Three later preparations yielded only material having m.p. 125°.

Ethyl α-amino-β-oxoadipate hydrochloride. Ethyl α-ethoxycarbonyl-β-oxoadipate (Eisner *et al.* 1950) was converted into *β*-oxoadipate by the method of Riegel & Lilienfeld (1945), or later, more conveniently, by the method of MacDonald & Stedman (1955). Ethyl *β*-oxoadipate (25 g.) in dry ether (200 ml.) was treated with a slight excess of ethyl nitrite and dry HCl, following the directions of Harington & Randall (1931) for *β*-oxoglutarate. At the end of the reaction the ethereal solution was concentrated until almost all the ether had been removed. To the residue, ether (20 ml.) was added, and the cooled solution was extracted three times with 100 ml. portions of 0·5M-Na_2CO_3 previously cooled to 2°. Each Na_2CO_3 extract was acidified to pH 3 as quickly as possible. The combined aqueous solution was extracted four times with ether. The combined ether extracts were dried and concentrated *in vacuo*. The residue was left in a vacuum desiccator over KOH for 18 hr.; it weighed 28 g.

Palladium–charcoal catalyst was prepared according to Harington & Randall (1931). The nitroso compound (28 g.) in 350 ml. of ethanol containing HCl (17·5 g.) was reduced catalytically at atmospheric pressure in the presence of palladium–charcoal (7·5 g.). Hydrogen uptake became very slow after 2 hr. and amounted to 78% of the theoretical. The catalyst was removed by filtration and washed with ethanol. The ethanolic solution on concentration *in vacuo* yielded *ethyl α-amino-β-oxoadipate hydrochloride* in a yield of 65% (based on wt. of the impure nitroso compound). Repeated recrystallization from ethanol–ether gave material with m.p. 155–156° (with decomp.). [Found: C, 44·6; H, 6·6; N (Kjeldahl), 4·9. $C_{10}H_{17}O_5N,HCl$ requires C, 44·9; H, 6·8; N, 5·2%.] On refluxing a solution of the hydrochloride in 5N-HCl for 3 hr. there was obtained in a yield of 80% a crystalline substance which was identified by m.p., mixed m.p. and chromatography as being δ-aminolaevulic acid hydrochloride. The diester had an R_F value of 0·58, the spot being located with the aid of ninhydrin, the picrate reagent (Shuster, 1956), and immediate reduction of $AgNO_3$ in the presence of NH_3. On

hydrolysis with HCl in the cold there appeared on chromatograms a spot with an R_F value of 0·39 giving colour reactions identical with those shown by the diester. Material with this R_F was also formed during the early stages of hydrolysis by 2N-HCl at 100°, whilst prolonged treatment produced mainly aminolaevulic acid. Pilot experiments with chromatography suggested that the new substance might be obtained in a high yield by treatment with 2N-HCl for 1 min. at 100°.

Ethyl hydrogen α-amino-β-oxoadipate hydrochloride (VII). A solution of the diester (0·9 g.) in 2N-HCl (5 ml.) was heated at 100° for 1 min. The solution was cooled and concentrated *in vacuo* to dryness; the residue was dried over KOH at 12 mm. for 18 hr. The resulting glass was dissolved with slight warming in dry ethanol; on addition of dry ether a crystalline substance was obtained with m.p. 151–154°, and which on chromatography appeared almost homogeneous; in addition to material of R_F 0·39, there was a trace of aminolaevulic acid (R_F, 0·23). Repeated recrystallization from ethanol–ether raised the m.p. to 156–157° (with decomp. but without marked evolution of gas) and led to an apparently complete removal of aminolaevulic acid. (Found: C, 39·9; H, 6·1; N, 5·7. $C_8H_{13}O_5N$,HCl requires C, 40·0; H, 5·8; N, 5·8%.) The mixed m.p. with the diester was 140–145°. The yield of *monoester* (VIII) was 55%. Electrometric titration of the ester (120 mg.) with 0·1N-NaOH indicated an apparent pK_1 4·05, and pK_2 6·4 (approx.). Heating the half-ester to 140° for 45 min. yielded material of unchanged m.p. and R_F value. The half-ester could easily be re-esterified to yield the original diester. A solution of the half-ester (50 mg.) in dry ethanol (5 ml.) containing HCl (1 g.) was allowed to stand at 20° for 3 days. Evaporation of the solution to dryness and crystallization of the residue from ethanol–ether yielded the original diethyl ester, as shown by chromatography and mixed m.p.

Ethyl methyl α-amino-β-oxoadipate hydrochloride (isomer *A*, IX). The half-ester (VII; 0·3 g.) was dissolved in 10 ml. of dry methanol containing HCl (6%, w/v). After standing at 20° for 1 hr. the solution was concentrated to dryness under reduced pressure, and the residue was dried over KOH *in vacuo*. The material was crystallized and recrystallized from methanol–ether. The yield was 60%. The m.p. of the *ethyl methyl ester* was 142·5°. (Found: C, 42·6; H, 6·0. $C_9H_{15}O_5N$,HCl requires C, 42·6; H, 6·3%.) Treatment of the mixed ester with benzyloxycarbonyl chloride under a variety of conditions did not yield crystalline material.

α-Ethyl δ-hydrogen α-benzyloxycarbamido-β-oxoadipate. The half-ester (VII; 0·46 g.) was dissolved in water at 4°, and to the solution was added in portions with intermittent shaking and cooling over 25 min. benzyloxycarbonyl chloride (0·4 ml.) and MgO (0·2 g.). A heavy precipitate formed during the reaction, and more precooled water (5 ml.) was added as required. The mixture was allowed to stand for 10 min. at 0° after the last addition and filtered. The precipitate was washed with cold, saturated $MgSO_4$ and ethyl acetate. On suspending the precipitate in a slight excess of 0·3N-HCl an oil appeared which soon crystallized. The material was dried and recrystallized successively from ethyl acetate–light petroleum (b.p. 60–80°) and chloroform–light petroleum (b.p. 60–80°). A further small amount of this material was obtained from the mother liquor of the magnesium-salt precipitate. The alkaline solution gave, on acidification, an oil which was extracted into ethyl acetate. Some crystalline material was obtained from this fraction which melted over a wide range. The amount was small and this fraction was discarded. The main product, which had m.p. 122°, was identified as *α-ethyl δ-hydrogen α-benzyloxycarbamido-β-oxoadipate.* [Found: C, 56·7; H, 5·6; N (Kjeldahl), 4·5; $C_{16}H_{19}O_7N$ requires C, 57·0; H, 5·8; N, 4·2%.] A portion (145 mg.) was dissolved in methanol (1 ml.) and titrated electrometrically, after addition of water (3 ml.), with 0·1N-NaOH. Alkali consumption to an apparent pH of 7·0 indicated an equivalent weight of 321 ($C_{16}H_{19}O_7N$ requires equiv. wt. 337). On further addition of alkali there appeared to be buffering in the region of pH 8–11, presumably caused by the ionization of an enolic group. After neutralization a further 14·0 ml. of 0·1N-NaOH (approx. 3 equiv.) was added and the solution was left for 2 days. Acidification gave a crystalline precipitate of m.p. 98–100°. Recrystallization and drying at 80° raised the m.p. to 122–123°, which was not lowered by admixture with δ-benzyloxycarbamidolaevulic acid. The material was hydrogenated with H_2 and Pd and the resulting amino acid was identified as δ-aminolaevulic acid. Treatment of the acylamidoadipate with 20 equiv. of 2N-NaOH gave the benzyloxycarbonyl derivative of glycine (m.p. and mixed m.p. 119–120°) in good yield; the corresponding derivative of aminolaevulic acid could not be isolated in this experiment. The product was hydrogenated and glycine was identified by chromatography.

Ethyl methyl α-amino-β-oxoadipate hydrochloride (isomer *B*, X). β-Oxoadipic acid (Eisner *et al.* 1950) (10 g.) in ether (250 ml.) was treated at 0° with an ethereal solution of diazomethane (2·5 equiv.). After removal of the solvent the residue was distilled. The main fraction distilled between 92° and 98° (0·02 mm. Hg) and was methyl β-oxoadipate. Bardham (1936) records b.p. 122° (0·5 mm. Hg). Yield was 45%. Nitrosation of the methyl ester with ethyl nitrite and reduction of the oximino group were carried out as described above for the corresponding ethyl ester; in particular catalytic reduction was carried out in 5% (w/v) ethanolic HCl. The yield of *ethyl methyl α-amino-β-oxoadipate hydrochloride* of m.p. 140–145° (with decomp.) was 60% (based on the weight of methyl β-oxoadipate). (Found: C, 42·9; H, 6·4; N, 5·6; Cl, 13·9. $C_9H_{15}O_5N$,HCl requires C, 42·6; H, 6·3; N, 5·5; Cl, 14·0%.) The mixed m.p. with isomer *A* (m.p. 142·5°, structure IX) was 127–128°. Hydrolysis by boiling with 5N-HCl for 1 hr. yielded CO_2, isolated as $BaCO_3$, and aminolaevulic acid hydrochloride, identified by mixed m.p. determination, chromatography and conversion into porphobilinogen by means of the specific dehydrase (Gibson, Neuberger & Scott, 1955). Hydrolysis with acid under mild conditions has so far not yielded material with properties expected for free α-amino-β-oxoadipic acid. Hydrolysis with alkali under a great variety of conditions produced, as measured by chromatography, mainly aminolaevulic acid and glycine.

Ethyl methyl α-benzyloxycarbamido-β-oxoadipate. The mixed ester (isomer *B*, 1·2 g.) dissolved in water (4 ml.) was treated at about 4° with benzyloxycarbonyl chloride (1·0 g.) and MgO (0·4 g.), both added in portions over 25 min. with intermittent shaking. The mixture was then allowed to stand for 25 min. and extracted with two portions of ethyl acetate, after the addition of 3 ml. of water. The combined ethyl acetate extracts were washed successively with water, saturated $KHCO_3$ and saturated NaCl. The residue was dissolved in warm benzene; on addition of light petroleum a crystalline precipitate appeared which was recrystallized from the same solvent mixture (m.p. 75–76°). The yield of *ethyl methyl α-benzyloxycarbamido-β-oxoadipate*

was 66%. (Found: C, 58·0; H, 6·1; N, 4·1. $C_{17}H_{21}O_7N$ requires C, 58·1; H, 6·3; N, 4·0%.) Hydrolysis of this material, with both acid and alkali under different conditions, was carried out. Apart from unchanged ester the only products which could be identified were aminolaevulic acid, glycine and their *N*-benzyloxycarbonyl derivatives.

BIOLOGICAL METHODS

Animals. Albino rats of the National Institute for Medical Research strain were used. All compounds were administered by intraperitoneal injection and, where urinary excretion of the injected compound or its metabolite was measured quantitatively, 0·9% NaCl (2 ml.) was injected intraperitoneally every 2 hr. for 8 hr.

Organism. A culture of *Rhodopseudomonas spheroides* was kindly supplied by Dr June Lascelles, Microbiology Unit, Department of Biochemistry, Oxford; the cells were grown in the light and harvested as described by Lascelles (1956). Incubations were at 35° under aerobic conditions, either in the light or in the dark, the detailed directions of Lascelles (1956) being followed. Cell-free extracts were obtained as follows: cells (0·5 g.) were harvested after 48 hr., washed with 0·15 M-KCl and ground in a precooled mortar containing alumina and 0·1 M potassium phosphate (pH 6·8; 15 ml.). The mixture was centrifuged (*c* 10 000 *g*) for 30 min. to yield 8 ml. of cell-free extract.

Liver homogenate. Rat liver was homogenized for 2 min. with 2 vol. of 0·15 M-KCl in a Potter–Elvehjem apparatus at a temperature close to 0°.

Isolation or estimation of compounds. Hippuric acid was isolated as described (Arnstein & Neuberger, 1951). Porphobilinogen was estimated by the method of Cookson & Rimington (1954) as modified by Berlin *et al.* (1956). Porphyrin formation in the microbiological experiments was measured by the method used by Lascelles (1956). Aminolaevulic acid was estimated by the picrate method of Shuster (1956).

Although free aminolaevulic acid requires heating with alkali and acetoacetate to give a product which yields a colour with Ehrlich's reagent (Granick & Vanden Schriek, 1955; Shuster, 1956), acetamidolaevulic acid was found to give a chromogen by alkali treatment, in the absence of acetylacetone or acetoacetate, in a similar manner to *N*-acetylglucosamine (Morgan & Elson, 1934). However, the colour was found to be less intense than that produced under the conditions described for aminolaevulic acid (Shuster, 1956). The latter method was therefore used for the estimation of the acetamido compound in the urine samples of rats given acetamidolaevulic acid. This was justified since the absence of coloration with picrate (Shuster, 1956) indicated that the urines did not contain free aminolaevulic acid. The relationship between acetamidolaevulic acid content and colour intensity was linear between 0·2 and 2·0 μmoles under the conditions of Shuster (1956).

Radioactivity measurements. Counting was done in 1 cm.^{2} disks at infinite thickness with an end-window Geiger counter.

RESULTS

Availability of certain N-acyl derivatives for glycine formation

The incorporation of radioactivity from succinyl- and *N*-β-carboxypropionyl-[α-^{14}C]glycine into hippuric acid and haem was compared with that from free [α-^{14}C]glycine (Table 1). The results supported the assumption that no incorporation took place until the labelled carbon was in the α-position in free glycine, and the data of Table 1 indicate the slight extent to which this process took place. To facilitate comparison, the corrected dilution co-

Table 1. *Availability of succinyl-*[α-^{14}C]*glycine* (*SG*) *or* β*-carboxypropionyl-*[α-^{14}C]*glycine* (*CPG*) *for formation of* [α-^{14}C]*glycine*

It is assumed that no incorporation of isotope took place until the test compounds had been converted into glycine (Gly), hence the percentage apparent hydrolysis is 100 × c.d.c. (Gly)/c.d.c. (observed); for definition see text. In Expts. 1–5, rats received sodium benzoate (0·2 m-mole/100 g.) intraperitoneally either at the same time as the labelled compound (Expts. 1 and 2) or 6 hr. later; hippuric acid was isolated from the urine voided during 24 hr. after the first injection. In Expts. 6 and 7, haemin was prepared from the blood of rats 5 days after injection of the labelled compound.

Expt. no	Labelled compound	Dose (m-mole/100 g.)	Specific activity (μc/m-mole)	Specific activity of compound isolated (μc/m-mole)	c.d.c.	Apparent hydrolysis to glycine (%)
			(*a*) Hippuric acid			
1	Gly	0·033	0·075	$6·04 \times 10^{-3}$	0·378 (0·356)*	(100)
2	CPG	0·033	$3 \times 0·075$	$3·16 \times 10^{-5}$	235	0·2
3	Gly	0·044	5·0	$3·92 \times 10^{-2}$	5·56 (5·24)*	(100)
4	SG	0·044	5·0	$8·71 \times 10^{-4}$	253	2·2
5	CPG	0·044	5·0	$7·91 \times 10^{-4}$	278	2·0
			(*b*) Haemin			
6	Gly	0·20	5·0	$1·21 \times 10^{-1}$	66	(100)
7	CPG	0·20	5·0	$1·54 \times 10^{-2}$	5500	1·2

* Data of Arnstein & Neuberger (1951).

efficients (C.D.C.; Arnstein & Neuberger, 1951) were calculated. C.D.C. $= Q(C_Q/C - 1)$, where Q is the dose of labelled compound, C_Q and C are the molar isotope contents of the compound administered and the compound re-isolated, respectively. For haemin, which contains eight glycine α-carbon atoms, C is one-eighth of the isotope content measured.

Availability of certain N-acyl derivatives for aminolaevulic acid formation

If hydrolysis of an *N*-acetylated derivative of δ-aminolaevulic acid were to take place *in vivo*, then porphobilinogen should be detectable in the urine (Berlin *et al.* 1956). In fact no porphobilinogen or free aminolaevulic acid was detectable after giving δ-succinimido-, δ-succinamido-, or δ-acetamido-laevulic acid; the rate of excretion of the last compound was measured (Fig. 1). Its excretion was rapid and almost quantitative; further evidence that unchanged acetamidolaevulic acid was being excreted was supplied by the fact that the urine, on heating with alkali in the absence of ethyl aceto-acetate, gave then a red colour with Ehrlich's reagent (see Experimental).

Pyrrole formation from esters of amino-oxoadipic acid

There was clear evidence that both the di- and the (α)mono-ethyl esters of α-amino-β-oxoadipic acid were hydrolysed and decarboxylated *in vivo*. Porphobilinogen formation from these two compounds was readily measurable (Table 2), but was much less than from aminolaevulic acid itself.

Urine samples from rats which had received the diester were subjected to chromatography on paper as described by Berlin *et al.* (1956). Spots corresponding to aminolaevulic acid (R_F 0·23) were detectable in urine samples produced 2, 4, 6 and 8 hr. after injection. In addition, spots corresponding to a monoethyl ester of amino-oxoadipic acid were detectable after 2 and 4 hr. but not later.

The availability of diethyl α-amino-β-oxoadipate for porphyrin formation was demonstrated with cell suspensions (Fig. 2) or cell-free extracts (Table 3) of *Rps. spheroides*. This organism has been shown by Lascelles (1956) to produce different amounts of porphyrins from any one substrate, according as incubation is in the light or in the dark.

The ability of whole cells to utilize the diester was largely lost when they were broken up (Table 3).

Table 2. *Excretion of porphobilinogen (PBG) after administration of esters of α-amino-β-oxoadipic acid to rats*

Diethyl amino-oxoadipate or the (α)mono-ethyl ester (0·15 m-mole/100 g.) was injected. At this dose level 1·7–1·8% of ALA given has been found to be converted into urinary PBG (Berlin *et al.* 1956).

Time after injection (hr.)	PBG excreted (% of dose)	
	After diester	After monoester
2	0·027	0·027
4	0·052	0·066
6	0·023	0·046
8	0·012	0·015
Totals	0·114	0·154

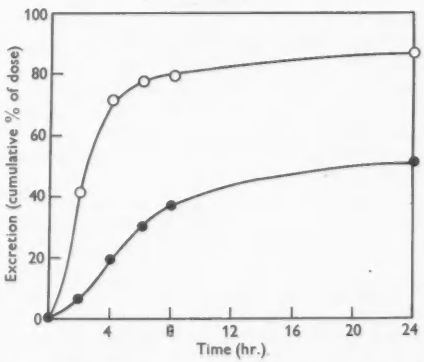


Fig. 1. Urinary excretion of δ-aminolaevulic acid (ALA) or of δ-acetamidolaevulic acid (AcALA). Rats were given either ALA or AcALA (0·2 m-mole/100 g.). No porphobilinogen or free ALA was detected in the urine of the rats given AcALA. Free ALA was determined by the picrate method and AcALA by the acetoacetate method. ○, AcALA after AcALA injection; ●, ALA after ALA injection.

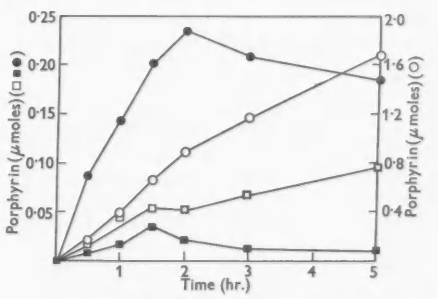


Fig. 2. Porphyrin formation from diethyl α-amino-β-oxoadipate, or from ALA, by *Rps. spheroides*. The incubation mixtures contained washed-cell suspension (2 mg. dry wt./ml.), potassium phosphate buffer (200 μmoles, pH 6·8) and substrate (20 μmoles) in a total vol. of 7 ml. Incubation was either in the dark or in the light, with shaking. At intervals, 1 ml. was removed and added to 3 ml. of 1·4 N-HCl; after centrifuging, the porphyrin content of the supernatant was measured as described. ○, ALA in the light; ●, ALA in the dark; □, diester in the light; ■, diester in the dark.

Table 3. *Porphyrin formation in a cell-free extract of* Rps. spheroides

Cell-free extract (1 ml.) was incubated either in the light or in the dark with potassium phosphate buffer (pH 6·8, 100 μmoles) and substrate (10 μmoles) in a total vol. of 2 ml.

	Time (hr.)			
	In the light		In the dark	
	1·25	18	1·25	18
Substrate	Porphyrin formed (mμmoles)			
Aminolaevulic acid	285	32	35	390
Diethyl α-amino-β-oxoadipate	6	7	6	21

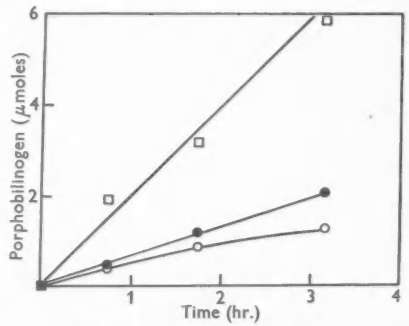


Fig. 3. Formation of porphobilinogen (PBG) in liver homogenates. The incubation mixture contained liver homogenate (4 ml.), 0·1 M potassium phosphate buffer (2 ml., pH 6·8) and substrate (20 μmoles). Incubation was at 37°, with gentle agitation. At intervals, 0·1 ml. was removed and added to 10% trichloroacetic acid (2 ml.) for assay of PBG after centrifuging. Substrates: □, ALA; ●, monoester, and ○, diester, of α-amino-β-oxoadipic acid.

Porphobilinogen formation from both the di- and the (α)mono-ethyl ester of amino-oxoadipic acid took place in rat-liver homogenates; as expected, the monoester was more effective than the diester, but both were rather less effective than aminolaevulic acid (Fig. 3).

DISCUSSION

It appears that *N*-succinyl- or β-carboxypropionylglycine are hydrolysed to a negligible extent, if at all, in the body. Tests with these two compounds labelled in the position corresponding to the α-carbon atom of glycine indicated (Table 1) that they enter into anabolic pathways of glycine to an extent which suggests that they may be excreted or oxidized at least as readily as they are hydrolysed. In oxidation their labelled α-carbon atoms would be only indirectly available for glycine formation. Such behaviour is in sharp contrast to that of *N*-formyl-, *N*-acetyl- and *N*-propionyl-glycine (Abbott 1942) and to similar derivatives of other amino acids (for review see Jackson & Chandler, 1939). A similar failure to hydrolyse *N*-succinyl and *N*-β-carboxypropionyl derivatives has been observed in succinimido- and succinamido-laevulic acids, but in this case the *N*-acetyl derivative is also not split in the body (Fig. 1); in contrast to the latter finding, Neuberger & Sanger (1943) found that ε-*N*-acetyllysine can replace the parent amino acid in deficient diets. The failure of the body to hydrolyse these two *N*-β-carboxypropionyl amino acids recalls the finding of Christman & Lewis (1921) that esterases will hydrolyse only the first ester group of dialkyl succinates, being apparently inhibited by the close proximity of the charged carboxyl group previously liberated.

Tables 2 and 3 and Figs. 2 and 3 show that diethyl or, better, (α)mono-ethyl α-amino-β-oxoadipate can give rise to δ-aminolaevulic acid both *in vivo* and, to a lesser extent, in cell-free biological systems. The observation that the diester gives rise to urinary porphobilinogen after injection into rats was also made by Shemin *et al.* (1955). Although these esters of amino-oxoadipic acid have been shown to give rise to aminolaevulic acid, porphobilinogen and porphyrins, it cannot be claimed on this evidence alone that the free acid is in fact a normal intermediate in biosynthesis, since similar findings would have resulted from spontaneous decarboxylation of this β-keto acid after saponification by an esterase.

SUMMARY

1. The diethyl ester and the (α)mono-ethyl (δ)hydrogen ester of α-amino-β-oxoadipic acid hydrochloride have been synthesized. The two ethyl methyl esters of this acid have also been prepared and identified. *N*-Benzyloxycarbonyl derivatives of certain of these compounds were prepared.

2. The hydrolysis of esters of amino-oxoadipic acid, or of the corresponding *N*-benzyloxycarbonyl derivatives, has been studied. It has not so far been possible to isolate the free amino acid. Under acid conditions the above-mentioned half-ester and aminolaevulic acid were the main products. Under alkaline conditions aminolaevulic acid and glycine, or their *N*-acyl derivatives, were the main nitrogenous products. δ-Benzyloxycarbamidolaevulic acid was also prepared from aminolaevulic acid hydrochloride; the compound occurs in two crystalline modifications.

3. δ-Acetamidolaevulic acid has been synthesized from aminolaevulic acid and was also prepared by reductive acetylation of δ-oximinolaevulic acid.

4. In contrast to simple unbranched *N*-acyl derivatives of glycine, *N*-β-carboxypropionyl- and *N*-succinyl-glycine are hydrolysed *in vivo* to a negligible extent only. Corresponding derivatives of aminolaevulic acid also are not hydrolysed, nor is, in this case, the *N*-acetyl amino acid. With the latter compound rapid excretion may account for the failure of hydrolytic systems to yield the free amino acid.

5. Both the diester and the (α)mono-ethyl ester of amino-oxoadipic acid have been shown to give rise to porphobilinogen or porphyrins in biological systems. These included rats, liver homogenates, and suspensions or cell-free extracts of *Rhodopseudomonas spheroides*. This is not, however, sufficient evidence to establish that amino-oxoadipic acid is the biological precursor of aminolaevulic acid.

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The Concentration of Glucose in Rat Tissues

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Paper chromatography has been used in the present investigation for the determination of the true glucose content of eight tissues of the rat. This method is more effective than older methods in separating glucose from other reducing substances in tissue extracts, but it does not appear to have been used before for systematic investigations of the glucose content of animal tissues, though Wallenfels, Bernt & Limberg (1953) used it for measuring glucose in blood. Previous authors have determined the yeast-fermentable reducing substances in some tissues, and the values obtained by chromatography in the present work are of the same order.

EXPERIMENTAL

Preparation of tissues. Male albino rats, weighing 105–160 g., were used, after having been kept without food for 20–28 hr. but supplied with water *ad lib.* The animals were killed instantaneously either by immersion in liquid oxygen or by decapitation. In the latter method tissues were put in liquid oxygen after a specified time. The time interval between death and freezing is given in the first column of Tables 2–9. The tissues were broken up in the liquid oxygen with a pair of pliers and quickly weighed on a torsion balance while still frozen. The weighed tissue (up to 450 mg.) was ground in an ice-cold mortar with acid-washed sand in 1 ml. of 0·3N-$Ba(OH)_2$ (Somogyi, 1945). The following additions were then made: 1 ml. of 0·3N-$Ba(OH)_2$, water (2 ml. minus an allowance for tissue water, taken to be 80% of the

tissue weight) and 2 ml. of $ZnSO_4$ (5% $ZnSO_4,7H_2O$, w/v). After the material had stood for 20 min. at room temperature it was filtered through a sintered-glass funnel (porosity grade 4). Blood was obtained from decapitated animals. It was heparinized and centrifuged. The plasma was stored for up to 2 hr. at 1–3° while the tissues were dealt with. For deproteinization 0·1 ml. of plasma was diluted to 2 ml. with water and 2 ml. of 0·3 N-$Ba(OH)_2$ and 2 ml. of $ZnSO_4$ were added.

Determination of total free reducing substances. The method of Nelson (1944) was applied to 1 ml. of filtrate of the material treated in this way. This procedure gave lower figures than were given by the method of Miller & Van Slyke (1936) or by deproteinization according to Folin & Wu (1919, 1922), followed by Nelson's method. It seems therefore to be more specific, and has the further advantage of giving good chromatographic separations. The deproteinization according to Cori, Closs & Cori (1933) with acidified $HgSO_4$ also proved suitable for subsequent chromatography, and gave about the same values, but it is more laborious.

A portion of the filtrate (1 ml.) was mixed with 1 ml. of copper mixture and 1 ml. of the arsenomolybdate reagent, as described by Nelson (1944). The spectrophotometer readings were made in a volume of 10 ml., usually at 660 mμ., but at 520 mμ. for higher concentrations. All readings were compared with standards treated identically.

Determination of glucose. A portion of the filtrate (2 ml.) was evaporated either at 40° by vacuum distillation or in a vacuum desiccator over $CaCl_2$ at 2 mm. Hg. The remaining filtrate (approx. 2 ml.) was also evaporated. The two residues were dissolved in 0·1 ml. of 0·001 N-HCl and quantitatively transferred (with one washing of 0·1 ml. of 0·001 N-HCl and one of 0·1 ml. of water) to 2 cm. strips of Whatman no. 1 paper. Up to 15 parallel 2 cm. strips were obtained from an 18¼ in. × 22½ in. Whatman paper sheet by cutting out segments of 1 cm. × 45 cm. so that the remaining strips were held together by a crossing strip at the top and bottom of the sheet. A stream of cool air was used to dry the solutions on the paper. The chromatographic solvent was *n*-propanol–ethyl acetate–water (7:1:2, by vol.) (Baar, 1954). The paper was developed by descending chromatography for 26–30 hr. and dried by warm air at 40–50°. The second strip was sprayed with benzidine–trichloroacetic acid (Bacon & Edelman, 1951) to locate the glucose. An area of about 2 cm. × 4 cm. corresponding to the glucose spot on the sprayed strip was cut out and extracted in 3 ml. of water at 60–70° for about 1 hr. The paper was partly disintegrated by vigorous shaking for a few seconds in the glass-stoppered tube. The cellulose fibres were then removed by filtration through sintered glass (porosity grade 4). Copper mixture (1 ml.) was added to 2 ml. of this filtrate and glucose was estimated colorimetrically as previously described. Glucose standards were run on parallel strips. Duplicate chromatographic estimations agreed within 4% if 20–200 μg. of glucose was present and within 7% if less than 20 μg. was present. The agreement between duplicates was within 10% when two pieces of tissue were analysed. Additions of 50–110 μg. of glucose to liver, kidney and brain were recovered within this limit of 10%. Results are expressed as mg. of glucose/100 g. of frozen tissue.

RESULTS

On average, 87% of the total reducing value for rat-blood plasma was found to be due to glucose (Table 1). A similar value was observed by Wallenfels *et al.* (1953) chromatographically for glucose in human-blood serum, and by earlier authors (Hiller, Linder & Van Slyke, 1925; Folin & Svedberg, 1926; Somogyi, 1927; Benedict, 1928; Molitor & Pollak, 1931) for yeast-fermentable substances in plasma or serum.

The values obtained for glucose and total reducing substances of various organs immediately after death, and a few minutes later, are given in Tables 2–9. As the results for the different time intervals after death were necessarily derived from different animals the variations observed represent not only the effect of differences in time but also of individual variability. The concentrations of glucose as well as

Table 1. *Total reducing substances and glucose in the blood plasma of rats*

Rat no.	Total reducing substances as glucose (mg./100 ml.)	Glucose (mg./100 ml.)	Glucose (% of total reducing substances)
28	61	59	97
10	82	67	82
8	76	71	93
12	111	85	77
30	124	110	89

Table 2. *Total reducing substances and glucose in the cerebral hemispheres of starved rats*

Rat no.	Time of freezing after death (min.)	Total reducing substances as glucose (mg./100 g.)	Glucose (mg./100 g.)	Glucose (% of total reducing substances)	Plasma glucose (mg./100 ml.)
1	0	19	—	—	—
		20	8	40	—
2	0	22	8	36	—
		20	8	40	—
3	0·75	22	—	—	90
4	0·75	18	—	—	—
5	1·5	12	7	58	60
6	1·5	14	6	43	60
7	2	11	1	9	—

Table 3. *Total reducing substances and glucose in the testis of starved rats*

Rat no.	Time of freezing after death (min.)	Total reducing substances as glucose (mg./100 g.)	Glucose (mg./100 g.)	Glucose (% of total reducing substances)	Plasma glucose (mg./100 ml.)
1	0	33	8	24	—
2	0	34	9	27	—
8	3	22	3	14	76
9	3	20	2	10	78
10	3·5	34	2	6	82
11	3·5	—	3	—	80

Table 4. *Total reducing substances and glucose in leg musle of starved rats*

Rat no.	Time of freezing after death (min.)	Total reducing substances as glucose (mg./100 g.)	Glucose (mg./100 g.)	Glucose (% of total reducing substances)	Plasma glucose (mg./100 ml.)
1	0	54	22	41	—
2	0	45	22	49	—
8	1·5	59	34	57	76
9	1·5	52	24	54	78
10	1·5	55	34	62	82
11	1·5	39	17	44	80
12	2·5	44	31	70	85
13	2·5	55	—	—	104

of the total reducing substances were found to be very low in the cerebral hemispheres (Table 2). Glucose averaged 8 mg./100 g. and total reducing substances 20 mg./100 g. in the animals immersed in liquid oxygen. There are no data in the literature on the concentration of glucose, as opposed to total reducing substances, in the brain. The total reducing substances reported in earlier publications [all values from animals under Amytal (5-ethyl-5-*iso*pentylbarbituric acid) anaesthesia] were all higher: 33–71 mg./100 g. was found in the brains of starved dogs (Kerr & Ghantus, 1936), 35–37 mg./100 g. in the brains of starved rabbits (Kerr & Ghantus, 1936; Kerr, Hampel & Ghantus, 1937; Gurdjahn, Webster & Stone, 1949), and 74 mg./100 g. in the brains of unstarved cats (Klein, Hurwitz & Olsen, 1946; Klein & Olsen, 1947). The lower values for total reducing substances found in the rat brain may be due to species differences, or to differences in procedure.

The following considerations are relevant to the question whether the glucose values found in the animals killed by immersion in liquid air are identical with those occurring in the living animal. Richter & Dawson (1948) measured the time required to freeze the whole brain of a 40 g. rat placed in liquid air. They found that the temperature remained nearly normal for 9–20 sec. and then dropped quickly. As rats weighing 100 g. were used in the present experiments a slower rate of cooling is expected. If an assumption is made on the cooling rate of the tissue the amount of glucose consumed by the tissue can be calculated from known Q_{O_2} values, conditions being aerobic. It is assumed that the tissue remained at body temperature for 30 sec. The highest value reported, Q_{O_2}, −26 (Krebs, 1950), corresponds to a glucose consumption of 6 mg./100 g./0·5 min. There is no reason for assuming that the glucose supply from the blood ceases immediately on cooling, and 6 mg./100 g./0·5 min. may therefore be regarded as the maximum disappearance rate. The actual rate is probably smaller. If the maximum figure is added to the observed value of 8 mg./100 g., a glucose concentration of 14 mg./100 g. is obtained. The results in tissue frozen up to 2 min. after killing indicate that the fall of tissue glucose is certainly not greater than 6 mg./100 g./0·5 min. At least as much of the reducing substances other than glucose as of glucose was found in brain.

Testis also gave low values for glucose and total reducing substances, namely 8 mg./100 g. for glucose, and 25 mg./100 g. for reducing substances other than glucose (Table 3). LePage (1946*a*, *b*) found that 40 sec. was required to freeze completely the abdominal organs of a 300 g. rat placed in liquid air. As the rate of glucose consumption of testis is lower than that of brain [Q_{O_2}, −12 (Krebs & Johnson, 1948)], the correction for post-mortem changes may be taken to be at most 3 mg./100 g. As in brain, the glucose value fell to a very low level within 2 min. The changes of the reducing substances other than glucose were smaller and less uniform than those for glucose.

Tables 4 and 5 show the amount of glucose in 100 g. of muscle and diaphragm. In skeletal muscle

22 mg./100 g. was found in the quickly frozen animal, and somewhat more in four out of five muscles frozen 1·5–3 min. after death. Diaphragm shows a similar post-mortem increase of free glucose. The glucose concentrations found are in general agreement with the concentrations of yeast-fermentable substances reported by Power & Clawson, 1928; Bischoff & Long, 1932; Trimble & Cary, 1931, and somewhat higher than those found with Cori's method (Cori *et al.* 1933). The value for total reducing substances of diaphragm agrees with data of Krahl & Cori (1947). Lower values found by Bornstein & Park (1953) are probably due to differences in technique.

Spleen showed glucose concentrations similar to those of muscle (Table 6): 22–23 mg./100 g. was found in the animal quickly frozen in liquid oxygen, decreasing to 6 mg./100 g. within 3·5 min. after death. The concentration of non-reducing substances was very low.

The average glucose value in the kidney (mainly cortex) was 51 mg./100 g. in the first minute (Table 7), and the value for reducing substances other than glucose was low. Within 3·5 min. after death the glucose level dropped to 15 mg./100 g., but the total reducing substances did not change. This suggests that glucose is rapidly converted into another reducing substance. The rapid changes after

Table 5. *Total reducing substances and glucose in the diaphragm of starved rats*

Rat no.	Time of freezing after death (min.)	Total reducing substances as glucose (mg./100 g.)	Glucose (mg./100 g.)	Glucose (% of total reducing substances)
14	0·5	58	25	43
15	1	45	24	53
16	1	44	—	—
17	1·5	49	32	65

Table 6. *Total reducing substances and glucose in the spleen of starved rats*

Rat no.	Time of freezing after death (min.)	Total reducing substances as glucose (mg./100 g.)	Glucose (mg./100 g.)	Glucose (% of total reducing substances)	Plasma glucose (mg./100 ml.)
1	0	23	22	96	—
2	0	24	23	96	—
10	1·5	15	13	87	82
18	2	34	25	73	92
19	2	17	15	88	90
12	3	10	8	80	85
13	3	17	14	82	104
8	3·5	9	6	67	76

Table 7. *Total reducing substances and glucose in the kidneys of starved rats*

Rat no.	Time of freezing after death (min.)	Total reducing substances as glucose (mg./100 g.)	Glucose (mg./100 g.)	Glucose (% of total reducing substances)	Plasma glucose (mg./100 ml.)
1	0	55	51	93	—
2	0	57	55	96	—
18	1	51	43	84	92
19	1	54	54	100	90
12	1·5	51	43	84	85
13	1·5	44	38	86	104
20	1·5	52	39	75	—
21	1·5	54	40	74	—
8	2·5	50	37	74	76
9	2·5	43	36	84	78
22	2·5	40	29	72	—
23	2·5	42	29	69	—
10	2·5	39	22	57	82
11	3·5	45	15	33	80

Table 8. *Total reducing substances and glucose in the lungs of starved rats*

Rat no.	Time of freezing after death (min.)	Total reducing substances as glucose (mg./100 g.)	Glucose (mg./100 g.)	Glucose (% of total reducing substances)	Plasma glucose (mg./100 ml.)
1	0	63	63	100	—
3	3	36	—	—	90
10	3	32	25	78	82
11	3	32	25	78	80

Table 9. *Total reducing substances and glucose in the livers of starved rats*

Rat no.	Time of freezing after death (min.)	Total reducing substances as glucose (mg./100 g.)	Glucose (mg./100 g.)	Glucose (% of total reducing substances)	Plasma glucose (mg./100 ml.)
1	0	93	88	95	—
2	0	90	85	95	—
24	0·5	99	85	86	—
25	0·75	99	93	94	—
8	0·75	84	82	98	76
9	0·75	85	75	88	78
10	0·75	80	73	91	82
11	0·75	72	71	99	80
26	2	198	132	67	—
27	2	182	160	88	—
12	2	270	189	70	85
13	2	252	256	102	104
3	2	209	173	83	90

Table 10. *Total reducing substances and glucose in rat tissues within one minute after death*

Average values calculated from Tables 2–9.

Tissue	Total reducing substances as glucose (mg./100 g.)	Glucose (mg./100 g.)	Non-glucose reducing substances as glucose (mg./100 g.)
Brain	20	8	12
Testis	34	9	25
Leg muscle	50	22	28
Diaphragm	49	25	24
Spleen	24	23	1
Kidney	54	51	3
Lung	63	63	0
Liver	88	82	6

death may be connected with the high respiration of this tissue. If glucose were the substrate of respiration 18 mg. would be used/100 g. of tissues in 1 min. (Q_{O_2}, −38).

In lung 63 mg. of glucose/100 g. of tissue was found in an animal killed by freezing (Table 8). Three minutes after decapitation 25 mg./100 g. was present. The value for reducing substances other than glucose was low.

In liver (Table 9) values for glucose and total reducing substances did not change within the first minute after death. The average was 82 mg./100 g. for glucose, and 88 mg./100 g. for the total reducing substances. Two minutes after death the concentrations of both the glucose and reducing substances other than glucose were substantially higher (see Table 9). An increase in sugar after death has been known since Dalton (1871) and has been ascribed to the breakdown of glycogen (Panormoff, 1893; Molitor & Pollak, 1931). Molitor & Pollak (1931) determined the yeast-fermentable substances in liver and found 83 mg./100 g. in unstarved dogs and 97 mg./100 g. in rabbits, values which are of the same order as the glucose values found chromatographically in rat liver within the first minute after death.

The results obtained from different organs are summarized in Table 10, which presents the average concentrations found within the first minute after death for all organs investigated.

The glucose levels of the tissue increase in the following ascending sequence: brain and testis, leg muscle, diaphragm and spleen, kidney, lung, liver. The levels of reducing substances other than glucose were higher than the glucose levels in brain and testis, about equal to the glucose level in muscle, and very low in the other four tissues.

DISCUSSION

The glucose levels of the eight rat tissues show remarkable differences, varying between an average of 8 mg./100 g. in brain and an average of 82 mg./100 g. in liver.

Low glucose values seem to parallel relatively high rates of glycolysis and relatively low vascularization, although spleen is again an exception. Gibson *et al.* (1946) assessed the blood content of dog brain and muscle at about 2% of the tissue weight, compared with 20% in kidney, lung and liver.

The glucose concentration as measured is the resultant of the concentrations in the different tissue spaces. The low glucose values for brain and testis suggest that the glucose concentration of the 'extracellular' space in these tissues is lower than that of plasma. As the size of the 'glucose space' is not known no precise calculations can be made.

The glucose content increased after death in liver and muscle, tissues known to contain appreciable amounts of glycogen, but fell in spleen, kidney and lung. The level of reducing substances other than glucose remained steady after death in those tissues which showed a relatively high initial value (brain, testis, muscle) and increased where the initial value was low (spleen, kidney, lung and liver).

SUMMARY

1. The true glucose content of eight rat tissues has been measured. Glucose was separated by paper chromatography and determined by the method of Nelson. The total reducing value was also determined.

2. When rats were killed by immersion in liquid oxygen, all the tissues except liver contained less glucose than did plasma, the value for brain and testis being only about one-tenth, and for muscle and spleen about a quarter, of the value for plasma.

3. The effects of delays in freezing the tissue after death were measured.

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Vitamin A_2 in Indian Fresh-water Fish-Liver Oils

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The possible existence of a new chromogen showing an absorption band at 693 mμ. in the colour test with antimony trichloride was first postulated by Heilbron, Gillam & Morton (1931). A little later, Edisbury, Morton & Simpkins (1937) designated this new chromogen vitamin A_2, on the strength of the very close similarity in the distribution of vitamin A_1 (620 mμ. band, colour test) in the liver oils of marine fishes and of the new chromogen in most fresh-water fishes. Simultaneously, Lederer & Rosanova (1937), in an examination of liver oils from fishes of Russian rivers, observed the predominance of the 693 mμ. band over the 620 mμ. band of vitamin A_1. Its importance in the visual

cycle of retinas of fresh-water fishes was recognized by the work of Wald (1937). The occurrence of vitamin A_2 in small amounts has now been shown in many oils from marine sources [e.g. Morton & Stubbs, 1948; Cama, Collins & Morton, 1951 (European waters) and Balasundaram, Cama, Sundaresan & Varma, 1956 (Indian waters)] and also to some extent in amphibia (Collins, Love & Morton, 1953).

After much earlier confusion, the elucidation of the structure of vitamin A_2 as the dehydro-vitamin A_1 formula was proved conclusively by synthesis (Farrar, Hamlet, Henbest & Jones, 1952). The dehydro-vitamin A_1 formula was suggested earlier by Morton, Salah & Stubbs (1947), mainly on the basis of the properties of crystalline vitamin A_2 aldehyde (retinene$_2$). Very recently an elegant conversion of vitamin A_1 into vitamin A_2 has been successfully carried out (Henbest, Jones & Owen, 1955) which makes the latter readily accessible. The aldehyde of vitamin A_1 (retinene$_1$) is treated with *N*-bromosuccinimide to obtain retinene$_2$, which on reduction with lithium aluminium hydride yields vitamin A_2.

Owing to its labile nature and, in part, to the scarcity of high-potency liver oils from fresh-water fishes, vitamin A_2 has not yet been obtained in a crystalline form. The spectroscopic properties (Shantz, 1948; Cama & Morton, 1953) and the biological potency of vitamin A_2, which is approximately 40% of that of vitamin A_1 (Shantz & Brinkman, 1950), have been determined with the purest preparation obtained.

Data on the content of vitamins A_1 and A_2 of liver oils from Indian fresh-water fishes measured spectrophotometrically are not available, and not much is known of the relative distribution of the vitamin. The present paper gives the result of investigations carried out on fishes in large fresh-water reservoirs in the States of Mysore and Madras. A short report of the work has already been published (Balasundaram, Cama, Sundaresan & Varma, 1955*a*, *b*).

EXPERIMENTAL

Fish-liver oils. Livers from fresh-water fishes were mainly obtained from the River Cauvery in the States of Mysore and Madras. A few samples were also obtained from Lake Hassargatta (Bangalore). The dietary habits of the fishes were either predatory (fish eating other fishes), or were based on subsistence on algae and vegetation. The two groups of feeders are marked (X) and (Y) in Table 1.

Potassium chromate and potassium dichromate. These were of Merck and AnalaR quality respectively, and were used to calibrate the Beckman spectrophotometer.

*Solvent. cyclo*Hexane, for spectroscopic use, was obtained from B.D.H. Ltd.

Light petroleum (b.p. 40–60°). This material, obtained from Burmah-Shell, was allowed to stand over $KMnO_4$ for a few days, washed, dried over $CaCl_2$ and twice distilled before use.

Chloroform (*B.P.*). This was allowed to stand over $CaCl_2$ and was filtered before use to remove any traces of ethanol.

Diethyl ether. This was allowed to stand over Na wire and freshly distilled before use over reduced Fe to remove any peroxide.

Alumina for adsorption chromatography. Alumina, specially prepared for chromatographic investigations (Merck), was used by weakening with known amounts of water, usually 5% (v/w), which was stirred in slowly under light petroleum.

Reagents. $SbCl_3$, acetic anhydride and KOH were B.D.H. laboratory reagents. Anhydrous reagent-grade Na_2SO_4 was generally used.

Micrometer syringe. For quantitative colour-test determinations, portions were accurately measured with the Agla micrometer syringe (Burroughs Wellcome Ltd.).

Absorption spectra and colour-test determination. The ultraviolet spectra of solutions were determined with the Beckman spectrophotometer, and in the absence of a Hilger–Nutting visual spectrophotometer antimony trichloride colour tests at specific wavelengths were done with the same instrument.

Calibration of the spectrophotometer. The spectrophotometer was calibrated with potassium chromate and dichromate (Cama *et al.* 1951).

Extraction and assay of oil. Fresh livers were ground with acid-washed sand and anhydrous sodium sulphate, and the oil was extracted with light petroleum (b.p. 40–60°). The combined extracts were reduced in bulk at low pressure and the ultraviolet spectra on the whole oil were obtained. A portion of the oil was saponified, and absorption spectra determinations and colour tests at 693 and 620 mμ. were carried out.

RESULTS

The contribution of vitamin A_2, in the ultraviolet spectrum of vitamin A_1 at 325 mμ., is appreciable. On the other hand, in the antimony trichloride colour test of vitamin A_2 at 693 mμ., the contribution of vitamin A_1 at 693 mμ. is almost negligible. The colour test at 693 mμ. is thus specific for the estimation of vitamin A_2 on the unsaponifiable fractions of fish-liver oils, and the reading could be evaluated for the ultraviolet absorption of vitamin A_2 at 350 mμ. The colour test for vitamin A_2 shows absorption at 620 mμ. equal to that at 693 mμ. divided by 2·45. From these figures, vitamin A_1 could be estimated by the colour test at 620 and 693 mμ., after applying proper corrections, and the reading is correlated to the equivalent ultraviolet absorption at 325 mμ. The method of estimation of vitamins A_1 and A_2 illustrated by Cama & Morton (1953) was followed. Only in a few cases, vitamin A_1 was estimated by the three-point correction method of Cama *et al.* (1951). Table 1 shows the vitamin A_1 and A_2 contents of liver oils from different species of fresh-water fishes, and Fig. 1 shows typical vitamin A_2 spectra of two liver oils of high potency from fresh-water fishes, examined after saponification.

Table 1. *Spectroscopic data from liver oils of some Indian fresh-water fishes*

Solvent used was *cyclo*hexane. *X*, predatory fish; *Y*, fish with vegetarian (alga) diet. $E^{1\%}_{1\,cm.}$ values for pure substances in *cyclo*hexane: crystalline vitamin A alcohol (326·5 mμ.) = 1745; $SbCl_3$ colour test (620 mμ.) = 5070 (Cama *et al.* 1951). Pure vitamin A_2 alcohol (351 mμ.) = 1320; $SbCl_3$ colour test (693 mμ.) = 3870 (Cama & Morton, 1953).

Reference	Name of species	Source	Percentage of oil from livers	$\lambda_{max.}$ of unsap. (mμ.)	$E^{1\%}_{1\,cm.}$ *			Corrected ($E^{1\%}_{1\,cm.}$ *)	
					At 351 mμ. vitamin A_2	At 693 mμ. vitamin A_2	At 620 mμ. vitamin A_1	351 mμ. vitamin A_2	326·5 mμ. vitamin A_1
A	'Wallago attu' (X)	Mysore, Madras	4	290, 351	127	307	157	106	11
B	*Ophiocephalus straitus* (X)	Hessaraghatta	4	290, 351	265	582	311	200	25
C	*Ophiocephalus straitus* (X)	Mysore	4	285, 351	99	252	102	89	Nil
D	*Macrones aor* (X)	Madras	6	290, 351	175·9	355	130	121	Nil
E	*Silundia* species (X)	Madras	5	287, 345	157	272	156	93	15
F	*Mystus* species (X)	Madras	5	290, 351	115·7	237	90	81	Nil
G	*Callichrous bimaculatus* (X)	Hessaraghatta	15	290, 345	259	618	329	211	26
H	*Ophiocephalus punctatus* (X)	Hessaraghatta	3	290, 345	44·1	62·7	40·6	21·4	5·1
I	*Notopterus notopterus* (X)	Madras	14	290, 330	53·1	86·5	44·5	29	3
J	*Notopterus notopterus* (X)	Hessaraghatta	2	330	23·4	36·3	50·7	12·4	12·3
K	*Cirrhina cirrhosa* (Y)	Madras	—	330	23	31·3	70	10·7	19·7
L	*Catla catla* (Y)	Madras	5	330	18·2	15	30	5·1	8
M	*Pungasius* species (X)	Madras	2	275, 340	8	11·1	7·8	3·8	1·1
N	*Mastacembelus armatus* (Y)	Hessaraghatta	10	275, 287, 345	1·7	3·55	1·85	1·2	0·13
O	*Barbus carnaticus* (Y)	Mysore	4	330	8·13	7·8	—	2·78	6·95†
P	*Barbus dubius* (Y)	Mysore	13	330	3·53	4·4	—	1·5	2·9†
Q	*Labeo* species (Y)	Mysore	15	290, 335	7·47	9·8	—	3·35	3·6†

* Expressed in terms of weight of original oil.

† Calculated by the three-point correction method (Cama *et al.* 1951).

Fig. 1. Absorption curves for unsaponifiable fractions of high-potency liver oils of fresh-water fishes. (Solvent: *cyclo*hexane). ●, 'Wallago attu'; ⊙, *Ophiocephalus straitus*.

Chromatography of oils. A large number of fractions were collected by chromatography of oil (about 0·2 g.) on a column of weakened alumina. The details of the original procedure (Barua & Morton, 1949) were followed. Each of the 10–20 ml. fractions collected was examined on the spectrophotometer, and those showing identical maxima were combined and their $E^{1\%}_{1\,cm.}$ values estimated. The results on two high-potency oils are recorded in Table 2.

Fraction I, obtained by chromatography of 'Wallago attu' oil (A), had marked absorption in the visible region of the spectrum. The fraction was reduced in volume and rechromatographed over alumina (without weakening). Distinct separation of a fine orange ring which travelled slowly down the chromatogram was effected; the pale greenish anhydro-vitamin A_1 with absorption at 350, 370 and 390 mμ. came down much faster. The orange ring had absorption maxima at 420, 450 and 470 mμ. characteristic of β-carotene, and behaved as such on the column.

Another interesting observation was the presence on the original column ('Wallago attu' oil) of a brownish-red band above the yellow vitamin A_2 alcohol band. This band was fast adsorbed on the column and did not move appreciably with light petroleum. The column was extruded and the band eluted with 50% ether–light petroleum mixture. When examined spectrophotometrically, the band had a main absorption maximum in the visible region at about 450 mμ., characteristic of lutein (?). Further work on the purification and characterization of the carotenoids is in progress.

Table 2. *Spectroscopic data from fractions obtained after chromatography of 'Wallago attu' (A) and* Ophiocephalus straitus *(B) oils on 5% weakened alumina*

Fractions	Solvent	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$ 350 mμ.* Oil A	Oil B	Component
1	Light petroleum (b.p. 40–60°)	350, 370, 390	0·121	0·101	Anhydro-vitamin A_1
2	Light petroleum (b.p. 40–60°)	290, 336·7, 345	1·72	0·454	—
3	Light petroleum (b.p. 40–60°)	285, 350	86·80	184·8	Vitamin A_2 esters
4	Ether–light petroleum (2:98, v/v)	330	14·0	14·6	Vitamin A_1 alcohol
5	Ether–light petroleum (5:95, v/v)	330, 350, 370, 390	2·25	—	Anhydro-vitamin A_2
6	Ether–light petroleum (10:90, v/v)	285, 350	12·65	17·0	Vitamin A_2 alcohol
7	Ether–light petroleum (15:85, v/v)	330, 355, 370	0·933	0·42	—
8	Ether–light petroleum (20:80, v/v)	336·7, 350	0·844	—	—
9	Ether	330	1·81	4·41	Oxidized vitamins A_1 & A_2 (?)
Gross $E^{1\%}_{1\,cm.}$ (350 mμ.) of the whole oil			127·0	243·8	
Total $E^{1\%}_{1\,cm.}$ (350 mμ.) of the fractions			121·13	221·78	

* Expressed in terms of weight of original oil.

DISCUSSION

It is evident from Table 1 that different species of fresh-water fish-liver oils vary in vitamins A_1 and A_2 potency to a considerable extent. The vitamin A_2 content of some of the oils examined is very high relative to oils previously reported in literature (Edisbury, Morton, Simpkins & Lovern, 1938; Gillam, Heilbron, Jones & Lederer, 1938; Lederer & Verrier, 1939; Cama *et al.* 1952; and Abdullah, Morcos & Salah, 1954). The oils which show high amounts of vitamin A_2 and little of vitamin A_1 are from fish having predatory habits, whereas oils from fish subsisting on vegetation have invariably low amounts of vitamin A_2. In this respect *Notopterus notopterus* (J), which is comparatively a very small fish, rarely growing to more than 2 lb. in weight, is an exception. *Punqusius* species (M) is also low, for no obvious reason.

Chromatographic investigations on high-potency oils show that most of the vitamin A_2 in the oils is present in the ester form without the simultaneous presence of vitamin A_1 ester (presence of 693 mμ. band and absence of 620 mμ. band with the antimony trichloride colour test on the vitamin A_2 ester fraction). Vitamin A_1 is present in the alcohol form only, and a small amount of vitamin A_2 alcohol is also present (Table 2).

The presence of β-carotene and lutein (?) in 'Wallago attu' liver oil is of interest. Similar observation on the fresh-water fish, brown trout, has also been reported earlier by Steven (1948). The biogenesis of vitamin A_2 is obscure, but it may well be that β-carotene is the precursor, as suggested by Morton & Creed (1939).

SUMMARY

1. Spectrophotometric determination of vitamins A_1 and A_2 on some Indian fresh-water fish-liver oils have been carried out.

2. Some species of fresh-water fishes show very high amounts of vitamin A_2 in their liver oils.

3. Chromatographic investigations indicate that most of the vitamin A_2 is present in the ester form and a small amount of vitamin A_2 alcohol is also present. Vitamin A_1, whenever present, is in the alcohol form only.

4. The occurrence of small amounts of β-carotene and lutein (?) in liver oils of fresh-water fishes is of interest.

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Fungal Detoxication

THE METABOLISM OF ω-(2-NAPHTHYLOXY)-*n*-ALKYLCARBOXYLIC ACIDS BY *ASPERGILLUS NIGER*

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Detoxication studies in higher animals have been extensively reviewed by Williams (1947), who has used the term 'detoxication' to cover all drug metabolism, whether or not associated with a decrease in toxicity. Its importance in other biological systems is now becoming increasingly apparent, e.g. in the development of drug resistance in bacteria (Albert, 1951, p. 168), in selective weed control (Wain, 1954) and in insecticidal action (Casida, 1955). The metabolism of toxic materials by fungi, however, has received little attention except in a number of specific instances (Rich & Horsfall, 1954; Byrde & Fielding, 1955). In view of its fundamental importance in fungicidal action, we have now commenced a systematic study of fungal detoxication.

The fungicidal activity of certain ω-aryloxyalkylcarboxylic acids (Crowdy, 1948) and the knowledge that they are capable of translocation in plants led to investigations of their possible value as systemic fungicides against plant disease (Crowdy & Wain, 1951; Byrde, Crowdy & Woodcock, 1953). Such studies were confined to assessments of their fungitoxicity both *in vivo* and *in vitro*, no attempt being made to investigate the biochemistry of the host–parasite–fungicide relationships.

From other sources, however, there is considerable evidence, both direct and indirect, that ω-aryloxyalkylcarboxylic acids undergo β-oxidation in plants, forming the corresponding aryloxyacetic acid or phenol according as the side chain possesses an even or an odd number of carbon atoms (Synerholm & Zimmerman, 1947; Fawcett, Ingram & Wain, 1954; Wain & Wightman, 1954; Luckwill & Woodcock, 1955). It has also been suggested (Holley, Boyle & Hand, 1950) that nuclear hydroxylation (in an unspecified position) may occur during the metabolism of 2:4-dichlorophenoxyacetic acid in plants.

Little is known of the metabolism of such acids by micro-organisms, however, though ω-phenyl-substituted fatty acids have been studied (Rittenberg & Ivler, 1952; Webley, Duff & Farmer, 1955). It was demonstrated by Webley *et al.* (1955), who used *Nocardia opaca* Waksman & Henrici, that, although acids with an odd number of carbon atoms in the side chain underwent β-oxidation to benzoic acid, *o*-hydroxyphenylacetic acid was the only product identified from the even-numbered acids. They considered that this acid was formed by a side reaction from phenylacetic acid, the remainder of which was metabolized further. Evans & Smith (1954) isolated a small motile bacterium from soil, which converted 4-chlorophenoxyacetic acid into 2-hydroxy-4-chlorophenoxyacetic acid and 4-chlorocatechol. A small Gram-negative motile rod was also isolated, which converted 2:4-dichlorophenoxyacetic acid into a phenolic acid, presumed, on chromatographic evidence, to be 6-hydroxy-2:4-dichlorophenoxyacetic acid. Brown & McCall (1955), however, have reported that this metabolite is not 2:4-dichloro-6-, 4-chloro-2- or 2-chloro-4-hydroxyphenoxy acetic acid.

The investigations described in this and subsequent papers were undertaken to explore the metabolism of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids by fungi. Some of the results formed the basis of a preliminary communication (Woodcock & Byrde, 1955).

EXPERIMENTAL

Materials and reference compounds

ω-(2-Naphthyloxy)-n-alkylcarboxylic acids. These were prepared as described by Pope & Woodcock (1954).

(6-Nitro-2-naphthyloxy)acetic acid. 6-Nitro-2-naphthol (4 g.) and ethyl bromoacetate (4 ml.) were refluxed overnight in a solution of sodium (0·52 g.) in ethanol (50 ml.). After the addition of 10% NaOH (10 ml.) the solution was refluxed for a further 1 hr., cooled, acidified and extracted with ether. Re-acidification of the bicarbonate-soluble material gave the required *acid*, which crystallized from aqueous ethanol in cream-coloured monoclinic prisms, m.p. 211–212°. (Found: C, 58·2; H, 3·8; N, 5·6. $C_{12}H_9O_5N$ requires C, 58·3; H, 3·6; N, 5·7%.) This compound was obtained by Shibata & Okuyama (1936), who gave m.p. 157–185°, but no analytical data. The *ethyl ester* crystallized from benzene in pale-yellow needles, m.p. 111–112°. (Found: C, 61·3; H, 4·7; N, 4·9. $C_{14}H_{13}O_5N$ requires C, 61·1; H, 4·7; N, 5·1%.)

Ethyl (6-amino-2-naphthyloxy)acetate. A solution of the ethyl ester (1·7 g.) in tetrahydrofuran (10 ml.) was shaken in hydrogen with Raney nickel until no further gas was absorbed. Removal of the solvent from the filtered solution and crystallization of the residual solid from ethanol gave the *amine* (1·3 g.) as nacreous plates, m.p. 80–81°. (Found: C, 68·1; H, 6·0; N, 5·7. $C_{14}H_{15}O_3N$ requires C, 68·6; H, 6·1; N, 5·7%.)

(6-Hydroxy-2-naphthyloxy)acetic acid. Ethyl (6-amino-2-naphthyloxy)acetate (1·3 g.) was diazotized at 0° in HCl solution with $NaNO_2$ (0·4 g.), and the solution poured into boiling 20% H_2SO_4 (100 ml.). The reddish solid product was extracted with ether and crystallized from aqueous methanol. Recrystallization of the *acid* from benzene gave prisms, m.p. 165–166°. (Found: C, 65·9; H, 4·8. $C_{12}H_{10}O_4$ requires C, 66·0; H, 4·6%.) The *methyl ester*, prepared in ethereal solution by the use of diazomethane, crystallized from benzene in prisms, m.p. 131–132°. (Found: C, 67·5; H, 5·3. $C_{13}H_{12}O_4$ requires C, 67·2; H, 5·2%.)

β-(6-Nitro-2-naphthyloxy)propionic acid. β-Propiolactone (2·5 g. = 1·5 equivalents) was added dropwise during 10 min. to a boiling stirred solution of 6-nitro-2-naphthol (4·7 g.) and NaOH (1 g.) in water (50 ml.). After the boiling solution had been stirred for a further 15 min. the solution was cooled, acidified and extracted with ether. The *acid*, isolated from the ethereal solution by shaking with $NaHCO_3$ solution and acidifying, was re-extracted with ether, the extract dried and the solvent removed. The residue crystallized from 50% aqueous methanol in long brown rectangular prisms, m.p. 163–164°. (Found: C, 59·5; H, 4·4; N, 5·3. $C_{13}H_{11}O_5N$ requires C, 59·8; H, 4·2; N, 5·4%.) The *ethyl ester*, prepared in the usual way, crystallized from ethanol in pale-yellow prisms, m.p. 110–111°. (Found: C, 62·6; H, 5·2; N, 4·9. $C_{15}H_{15}O_5N$ requires C, 62·3; H, 5·2; N, 4·8%.)

Ethyl β-(6-amino-2-naphthyloxy)propionate. This *ester* was prepared from the appropriate nitro ester as described above for the corresponding acetate. It crystallized from ethanol in colourless plates, m.p. 86–87°. (Found: C, 69·7; H, 6·7; N, 5·6. $C_{15}H_{17}O_3N$ requires C, 69·5; H, 6·6; N, 5·4%.)

β-(6-Hydroxy-2-naphthyloxy)propionic acid. A solution of the aminopropionic ester (2·3 g.) in a mixture of conc. HCl (5 ml.) and water (20 ml.) was diazotized at 0–5° by the gradual addition of $NaNO_2$ (0·7 g.) dissolved in a little water. After being stirred for 1 hr. the mixture was poured into boiling 20% H_2SO_4 (200 ml.) and cooled, and the mauve-coloured product collected, washed with water and dried at 100° (1·7 g., m.p. 155–180°). Repeated crystallizations of the *acid* from aqueous methanol (charcoal) finally gave prismatic plates, m.p. 194–195°. (Found: C, 67·3; H, 5·3. $C_{13}H_{12}O_4$ requires C, 67·2; H, 5·2%.) The *methyl ester* prepared in ethereal solution by the use of diazomethane, crystallized from benzene in large prismatic plates, m.p. 152–153°. (Found: C, 68·0; H, 6·0. $C_{14}H_{14}O_4$ requires C, 68·3; H, 5·7%.) The *p-nitrobenzyl ester*, prepared by refluxing in ethanolic solution, crystallized from acetone in prisms, m.p. 175–176°. (Found: C, 65·5; H, 4·3. $C_{20}H_{17}O_6N$ requires C, 65·4; H, 4·6%.)

γ-(6-Nitro-2-naphthyloxy)butyronitrile. 6-Nitro-2-naphthol (7·7 g.) was refluxed for 5 hr. with γ-bromobutyronitrile (8·2 ml.) in a solution of sodium (0·95 g.) in ethanol (50 ml.). After most of the ethanol had been distilled off the mixture was cooled, diluted with water and the *nitrile* extracted with ether. The ethereal solution was washed with dilute NaOH solution and then water, dried and the solvent removed. The residual solid crystallized from methanol in large orange-red plates, m.p. 99–99·5°. (Found: C, 65·5; H, 4·7; N, 10·8. $C_{14}H_{12}O_3N_2$ requires C, 65·6; H, 4·7; N, 10·9%.)

γ-(6-Nitro-2-naphthyloxy)-n-butyric acid. The nitrile (6·7 g.) was refluxed with water (50 ml.), conc. H_2SO_4 (30 ml.) and ethanol (100 ml.) for 18 hr. After the excess of ethanol had been distilled off, the solution was diluted with water and extracted with ether. The *acid* (0·45 g.) isolated with $NaHCO_3$ from the ethereal solution crystallized from aqueous ethanol in minute brown prisms, m.p. 184–185°. (Found: C, 61·0; H, 4·9; N, 5·2. $C_{14}H_{13}O_5N$ requires C, 61·1; H, 4·7; N, 5·1%.) Evaporation of the residual ether solution gave the *ethyl ester* (6·7 g.) m.p. 53–58° (67–68° after recrystallization from aqueous methanol). (Found: C, 63·5; H, 5·6; N, 4·7. $C_{16}H_{17}O_5N$ requires C, 63·4; H, 5·6; N, 4·6%.)

Ethyl γ-(6-amino-2-naphthyloxy)-n-butyrate. A solution of the nitro ester (2·3 g.) in tetrahydrofuran was shaken with Raney nickel in an atmosphere of hydrogen until no further uptake of gas was observed. Removal of the solvent gave the *amine*, which crystallized from aqueous ethanol in large nacreous plates, m.p. 86–87°. (Found: C, 70·6; H, 6·9; N, 5·2. $C_{16}H_{19}O_3N$ requires C, 70·3; H, 6·95; N, 5·1%.)

γ-(6-Hydroxy-2-naphthyloxy)-n-butyric acid. A solution of the amino-ester (1·9 g.) in HCl (5 ml.) and water (20 ml.) was diazotized at 0–5° by the dropwise addition of $NaNO_2$ (0·55 g.) dissolved in water (2 ml.). After stirring for 1 hr., the material was hydrolysed by adding the solution to boiling 20% H_2SO_4 (200 ml.). The product, collected after cooling, consisted of a mixture of the required acid and its ethyl ester; hydrolysis was completed by heating for a further 2 hr. with 10% NaOH (10 ml.). After cooling and acidifying, the *acid* was isolated with ether and washed with water, the ethereal solution dried and the solvent removed. The acid crystallized from aqueous methanol in aggregates of rectangular prisms, m.p. 157–158°. (Found: C, 68·0; H, 5·7. $C_{14}H_{14}O_4$ requires C, 68·3; H, 5·7%.)

Methods

Replacement culture technique and isolation of metabolites. Cultures of *Aspergillus niger* van Tiegh. (Mulder strain) were grown on the following medium, based on that described by Nicholas & Fielding (1951): glucose, 50 g.; KNO_3, 5 g.; KH_2PO_4, 2·5 g.; $MgSO_4,7H_2O$, 1 g.; Na_2SO_4, 1 g.; water, 1 l. The medium was sterilized in bulk (5 lb./sq.in. for

25 min.), and after addition of trace elements $FeCl_3,6H_2O$ (20 mg.), $ZnSO_4,7H_2O$ (10 mg.), $MnSO_4,4H_2O$ (3 mg.), $Na_2MoO_4,2H_2O$ (1·5 mg.) and $CuSO_4,5H_2O$ (1 mg.) was inoculated with a heavy spore suspension of *A. niger* and dispensed in sterile 20 oz. medicinal 'flats' (100 ml. each) or in penicillin culture flasks (500 ml. each).

After incubation at 25° for 3 days, the medium was poured off from the fungal mat, which was then washed with distilled water. It was replaced by a solution (or suspension, for acids of low solubility) of the test acid (0·001 M) in 0·01 M-Na_2HPO_4, the pH of which was approx. 8·1. After a further 7 days' incubation at 25°, the substrate was removed, strained through muslin and continuously extracted with ether for 18 hr. The ethereal solution was then shaken successively with saturated $NaHCO_3$, dilute NaOH and then water. Acidic products were isolated with ether after acidifying the $NaHCO_3$ solution and examined chromatographically as described below.

Sporulation was usually beginning when the new substrate was introduced, and marked pigmentation was apparent by the following day. In one experiment when fungal growth was poor and no pigmentation occurred, the usual metabolites could not be isolated.

Chromatographic examination of ether extracts. The ether extracts were evaporated to dryness, and the residue was dissolved in ether–methanol (4:1) (2·5 ml./l. of initial aqueous substrate). Samples (9·5 μl.), together with known compounds as required, were applied to Whatman no. 1 paper by means of a capillary pipette, and the chromatogram was developed overnight at laboratory temp. by downward solvent flow. The solvents used were *n*-butanol–ethanol–3 N ammonia (4:1:5) and *n*-butanol–acetic acid–water (4:1:5). The former gave best resolution, and was used for routine examinations.

After being dried at laboratory temperature, chromatograms were exposed under ultraviolet light (wavelength of maximum emission, 2539 Å) and fluorescent spots marked. They were subsequently sprayed with one of the following reagents: (1) *p*-nitrobenzenediazonium fluoroborate, as a 1% (w/v) solution in 20% (w/v) aqueous sodium acetate, for the detection of phenolic compounds as coloured spots (Freeman, 1952). If the papers were subsequently exposed to ammonia vapour while still moist, unhydroxylated acids showed as pale spots on a pink background. (2) Bromocresol green as a 0·04% solution in 95% (v/v) aqueous ethanol, adjusted to pH 5·5 (Lugg & Overell, 1948), for the detection of acids. This reagent was not suitable for papers developed in the ammoniacal solvent.

Where chromatographic evidence indicated the presence of only one metabolite, it was often possible to isolate it directly from the extracts by conventional methods. Where the presence of two major metabolites was indicated, however, and difficulty was encountered in isolating them, large-scale chromatographic separation on Whatman no. 3 MM paper was carried out. Bands fluorescing under ultraviolet light were marked and a marker strip from the edge of each sheet was sprayed with reagent (1). The appropriate bands on the main sheets were then cut out and eluted overnight with methanol. After evaporation of the solvents the residue was acidified with a few drops of conc. HCl and the acid extracted with ether.

In vitro *fungitoxicity.* For comparisons of fungitoxicity the medium already described was modified by increasing the KH_2PO_4 concn. to 3·4 g./l. (0·025 M) to ensure adequate buffering. The pH was adjusted to approx. 2·2 by the addition of H_3PO_4, the final phosphate concentration being 0·05 M.

The medium was sterilized in bulk, inoculated with a heavy spore suspension of *A. niger*, and dispensed in 50 ml. lots in 100 ml. conical flasks. To each was added 0·5 ml. of an ethanolic solution of the acid being tested; after thorough agitation, three replicates (16 ml. each) were dispensed into 4 oz. medicinal 'flats'. A control treatment, to which 0·5 ml. of ethanol alone was added, was included.

After incubation of the bottles on their sides for 6 days at 25°, the fungal mats were harvested, washed with distilled water, dried between sheets of coarse filter paper and then at 80° for 2–3 days. Mycelial dry weights were determined after cooling in a desiccator.

For statistical analysis the data (y) were transformed to x values, where $x = \log(y+1)$, since it was found that there was an approx. linear relationship between $\sqrt{[\text{variance}\ (y)]}$ and $\bar{y}$ (Anscombe, 1948).

RESULTS

Identification of metabolites

The metabolism of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids by *A. niger* gave hydroxy acids which were detected by their characteristic deep-pink colour when papers were sprayed with reagent (1). The identification of the principal metabolites is summarized in Table 1. Chromatographic examination of $NaHCO_3$-insoluble extracts yielded negative results in all cases.

Metabolism of 2-naphthyloxyacetic acid. The acidic material extracted from the fungal substrate after incubation with 2-naphthyloxyacetic acid was a light-brown amorphous powder, m.p. 148–150°, which, after several crystallizations from water (with charcoal), gave prismatic plates, m.p. 163–164°, not depressed by admixture with (6-hydroxy-2-naphthyloxy)acetic acid. (Found: C, 65·8; H, 4·3%.) The *methyl ester*, prepared as described above, crystallized from benzene in rosettes of prisms, m.p. 132–133°, not depressed by admixture with the authentic methyl ester. (Found: C, 67·5; H, 5·1%.)

Metabolism of β-(2-naphthyloxy)propionic acid. The acidic material extracted from the fungal substrate after incubation with β-(2-naphthyloxy)-propionic acid had m.p. 175–185°. Several recrystallizations from aqueous ethanol (with charcoal) gave plates, m.p. 193–195°, not depressed by admixture with β-(6-hydroxy-2-naphthyloxy)propionic acid. (Found: C, 67·4; H, 5·4%.) The *methyl ester*, prepared as described above, crystallized from benzene in plates, m.p. 147–150°, not depressed by admixture with the authentic methyl ester, m.p. 152–153°.

Metabolism of γ-(2-naphthyloxy)-n-butyric acid. Although 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids usually gave only single phenolic products, the presence of more than one phenolic spot was occasionally apparent with the

Table 1. *Identification of principal metabolites of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids incubated with* A. niger

Substrate [ω-(2-naphthyloxy) derivative of the acid shown]	Principal metabolite	R_F value*	Identification
Acetic acid	(6-Hydroxy-2-naphthyloxy)acetic acid (I)	0·30	Mixed m.p. and mixed m.p. of methyl ester with authentic specimens
Propionic acid	β-(6-Hydroxy-2-naphthyloxy)propionic acid (II)	0·46	Mixed m.p. and mixed m.p. of methyl ester with authentic specimens
n-Butyric acid	(I)	0·31	Mixed m.p. with authentic specimen
n-Valeric acid	(II)	0·46	Mixed m.p. with authentic specimen
n-Hexanoic acid	(I)	0·31	Mixed m.p. with authentic specimen
n-Heptanoic acid	(II)	0·45	Mixed m.p. with authentic specimen
n-Octanoic acid	(I)	0·30	R_F value
n-Nonanoic acid	(II)	0·45	Mixed m.p. with authentic specimen
n-Decanoic acid	(I)	0·30	R_F value
n-Dodecanoic acid	(I)	0·32	R_F value

* In *n*-butanol–ethanol–3N ammonia (4:1:5).

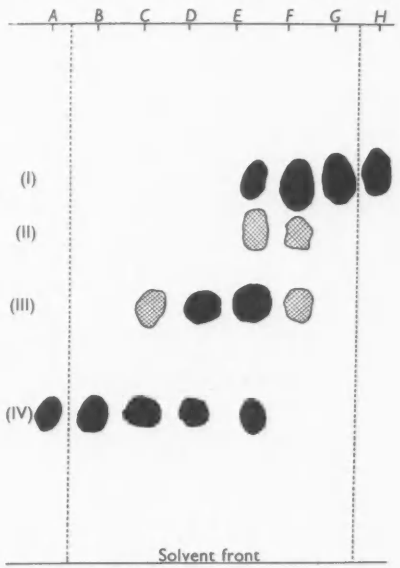


Fig. 1. Tracing of chromatogram of extracts from samples taken at intervals from γ-(2-naphthyloxy)-*n*-butyric acid incubated with *A. niger*. *A*: authentic γ-(2-naphthyloxy)-*n*-butyric acid. *B–G*: samples taken after 1, 2, 4, 8, 24 and 72 hr. respectively. *H*: authentic (6-hydroxy-2-naphthyloxy)acetic acid. All spots showed purple fluorescence under ultraviolet light (2539 Å). Spots (I), (II) and (III) gave deep-pink colour with *p*-nitrobenzenediazonium fluoroborate. Faint spots shaded.

higher members of this series. γ-(2-Naphthyloxy)-*n*-butyric acid was therefore selected for a more detailed study. Samples (25 ml.) from each of two flasks, initially containing 500 ml. of substrate, were taken after intervals of 1, 2, 4, 8, 24 and 72 hr. After the usual extraction procedure the acidic material was examined chromatographically together with the appropriate known compounds. The results are illustrated diagrammatically in Fig. 1. The gradual conversion of the butyric acid (IV) into (6-hydroxy-2-naphthyloxy)acetic acid (I) is obvious. Large-scale paper-chromatographic separation of acidic extracts obtained after 6 hr. enabled compound (III) (R_F 0·52) to be isolated. It crystallized from water and had m.p. 145–150°, not depressed by admixture with γ-(6-hydroxy-2-naphthyloxy)-*n*-butyric acid, m.p. 157–158°. The diagram also indicates the presence of a second, transient and less clearly marked intermediate (II) (R_F 0·39) between γ-(6-hydroxy-2-naphthyloxy)-*n*-butyric acid and (6-hydroxy-2-naphthyloxy)acetic acid, but no attempt was made to isolate this compound.

The conversion of the butyric acid (IV) into (6-hydroxy-2-naphthyloxy)acetic acid (I) was apparently unaffected by the presence of β-(2-naphthyloxy)propionic acid or 2:6-dihydroxynaphthalene.

Hydroxylation with a model system

Hydroxylation was further investigated with the ascorbic acid system developed by Udenfriend, Clark, Axelrod & Brodie (1954), which under physiological conditions of temperature and pH hydroxylates aromatic substances in a manner shown to be closely analogous to that occurring *in vivo*. A solution of 2-naphthyloxyacetic acid (0·12 g.), ascorbic acid (0·25 g.), $FeSO_4,7H_2O$ (0·04 g.) and sodium ethylenediaminetetraacetate (0·23 g.) in 0·2M phosphate buffer pH 6·8 (30 ml.) and acetone (10 ml.) was maintained at 37° for 2 hr. during the passage of a stream of air. The acidified solution was then extracted with ether and acidic substances were separated from other organic material with $NaHCO_3$ solution. Chromatographic examination of the bicarbonate-soluble fraction

showed the presence of unchanged 2-naphthyloxyacetic acid (R_F 0·63), (6-hydroxy-2-naphthyloxy)acetic acid (R_F 0·34) and an unknown substance giving a dark-brown spot with diazotized *p*-nitraniline (R_F 0·44). The bicarbonate-insoluble fraction gave only one spot (bright orange, R_F 0·97), which appears to be β-naphthol.

In vitro *fungitoxicity of unsubstituted acids*

Table 2 summarizes the results of toxicity tests with the unsubstituted acids against *A. niger* at pH 2·2. The pK_a value of 2-naphthyloxyacetic acid, determined experimentally, was 3·7, and a reliable comparison of inherent toxicity is therefore obtained at pH 2·2 where the acids are predominantly undissociated and thus more active (Simon & Blackman, 1949).

Table 2. *Fungistatic activity against* A. niger *of acids of the general formula* $C_{10}H_7.O.[CH_2]_n.CO_2H$ *at a concn. of* 10^{-4} M

n	Mean mycelial wt. (mg.)	Mean of transformed data*
1	335·4	2·53
2	5·9	0·66
3	5·3	0·76
4	28·3	1·33
5†	225·4	2·35
Control	295·2	2·47

Least significant difference between means of transformed data: (P = 0·05), 0·39.

* After applying the transformation $x = \log(y+1)$ (see text).

† No significant inhibition of growth for values of *n* from 6 to 8.

Relative toxicity of hydroxylated and unsubstituted acids

In preliminary experiments, provisional dosage–response lines for the unsubstituted and the corresponding hydroxylated acids were not parallel, and it was therefore impossible to obtain an absolute figure for relative potency (Finney, 1952, p. 65). The data of Table 3, however, show that the overall growth of the fungus in the presence of three fungistatic concentrations of 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids was significantly less than that in the presence of the corresponding hydroxylated acids at concentrations eight times higher.

DISCUSSION

The most significant feature of the present work on the metabolism of the ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids is their nuclear hydroxylation, together with β-oxidation of the side chain. Though this substitution of a hydroxyl group in position 6 in preference to position 1 is chemically unusual, hydroxylation in both positions with 2-naphthylamine has been reported with rat-liver slices (Booth, Boyland & Manson, 1955), and in the animal body (Boyland, Manson, Sims & Williams, 1955). The same substitution was shown by the former workers to be possible with the ascorbic acid–$FeSO_4$

Table 3. *Effect of 6-hydroxylation on the fungistatic activity of 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids against* A. niger

2-Naphthyloxyacetic acid			(6-Hydroxy-2-naphthyloxy)acetic acid		
Concn. ($\times 10^{-4}$ M)	Mean mycelial wt. (mg.)	Mean of transformed data*	Concn. ($\times 10^{-4}$ M)	Mean mycelial wt. (mg.)	Mean of transformed data*
2·50	0·0	0·000	20·0	73·5	1·853
1·75	2·7	0·367	14·0	140·1	2·140
1·25	84·0	1·470	10·0	148·6	2·167
Overall mean	28·9	**0·612**	Overall mean	120·7	**2·053**
β-(2-Naphthyloxy)propionic acid			β-(6-Hydroxy-2-naphthyloxy)propionic acid		
Concn. ($\times 10^{-4}$ M)	Mean mycelial wt. (mg.)	Mean of transformed data*	Concn. ($\times 10^{-4}$ M)	Mean mycelial wt. (mg.)	Mean of transformed data*
1·25	0·0	0·000	10·0	2·1	0·477
0·88	0·0	0·000	7·0	5·0	0·753
0·63	2·3	0·410	5·0	45·4	1·663
Overall mean	0·8	**0·137**	Overall mean	17·5	**0·964**

Least significant difference between individual means of transformed data: (P = 0·05), 0·562; (P = 0·01), 0·761; (P = 0·001), 1·019.

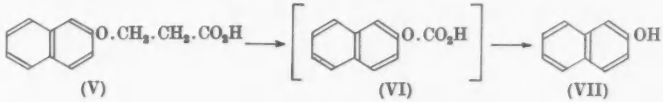
Least significant difference between overall means of transformed data: (P = 0·05), 0·324; (P = 0·01), 0·439; (P = 0·000), 0·588.

Comparable mean wt. of controls = 234·4 mg. (mean of transformed data = 2·370).

* After applying the transformation $x = \log(y+1)$ (see text).

reagent of Udenfriend *et al.* (1954). This system has also been shown by Dalgliesh (1955) to hydroxylate phenylacetic acid in the *ortho-* and *para*-positions, and we have found that 2-naphthyloxyacetic acid may under these conditions give another hydroxy acid in addition to (6-hydroxy-2-naphthyloxy)-acetic acid. Identification of the unknown acid has not been possible, however, owing to the inaccessibility of (1-hydroxy-2-naphthyloxy)acetic acid. Synthetic routes to acids of this type are being currently explored.

Although those acids having an even number of carbon atoms in the side chain were successively β-oxidized to (6-hydroxy-2-naphthyloxy)acetic acid, as might be expected from earlier work with these compounds in certain plants, the corresponding acids with an odd number of carbon atoms in the side chain provided an anomaly. It has previously been postulated that, in plants, β-(2-naphthyloxy)-propionic acid (V) would be oxidized via the hypothetical carbonate (VI) to β-naphthol (VII). The latter compound has in fact been detected chromatographically after incubation of *Sclerotinia laxa* Aderh. & Ruhl. with β-(2-naphthyloxy)-propionic acid (Byrde & Woodcock, unpublished work). It was to be expected, therefore, that the corresponding end-product with *A. niger* would be 2:6-dihydroxynaphthalene, but no diazo-positive material was observed when the alkali-soluble (as distinct from the bicarbonate-soluble) fraction was chromatographed, nor was there any evidence of 2:6-naphthaquinone formation. An unambiguous synthesis has confirmed that the product isolated was β-(6-hydroxy-2-naphthyloxy)propionic acid.



(V) (VI) (VII)

The reason for the failure of β-oxidation at this stage is not yet clear. Though the possibility that β-oxidation does initially occur, forming successively 2:6-dihydroxynaphthalene and then 2:6-naphthaquinone, is an attractive one, we have been unable to obtain any evidence that the former compound can interfere with this process. Moreover, incubation of γ-(2-naphthyloxy)-*n*-butyric acid with *A. niger* in the presence of β-(2-naphthyloxy)propionic acid has shown that the γ-compound is converted into (6-hydroxy-2-naphthyloxy)-acetic acid. Inhibition of the β-oxidation process by any metabolic product of β-(2-naphthyloxy)-propionic acid thus seems most unlikely. Ring closure of the propionic acid to form a substituted γ-pyrone would presumably prevent β-oxidation, but again there is no evidence to support this suggestion.

Chromatographic examination of the metabolism of γ-(2-naphthyloxy)-*n*-butyric acid (IV) (Fig. 1) indicated that nuclear hydroxylation occurred rapidly and took precedence over β-oxidation. (6-Hydroxy-2-naphthyloxy)acetic acid was also formed by incubation of γ-(6-hydroxy-2-naphthyloxy)butyric acid (III) with *A. niger*. The possibility of limited metabolism by the alternative pathway (β-oxidation followed by hydroxylation) cannot be excluded. Compound (II) is presumably an intermediate in the β-oxidation of (III).

In the homologous series $C_{10}H_7.O.[CH_2]_n.CO_2H$, where $n = 1$–9, there are therefore two principal phenolic end-products, and the fungitoxicity of each has been shown to be less than that of the corresponding unsubstituted acid. The hydroxylation of 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids thus appears to be a true detoxication mechanism (Williams, 1947, p. 241). Although this may be due to the effect of the hydroxyl group in lowering the 'lipophilic–hydrophilic ratio' (Veldstra & Booij, 1949) the difference in slope of the dosage–response lines observed in preliminary experiments implies a different mode of fungistatic action (Finney, 1952, p. 124).

The toxicity of the unsubstituted acids followed the normal pattern found in homologous series (Ferguson, 1939; Albert, 1951, p. 32). Following maximal values at $n = 2$–3, toxicity fell off markedly as the series was further ascended. It is thus possible that, at a certain length of side chain, the toxicity of the acids may in fact be lower than that of the hydroxylated end-product. Above $n = 3$ there was also some slight indication of an alternation in toxicity between odd and even values of n, suggesting that β-oxidation may be occurring.

The mechanism of hydroxylation in biological systems is obscure. Brodie *et al.* (1955) have suggested that it is brought about by non-specific peroxidase-type enzymes, the hydrogen peroxide arising from the enzymic breakdown of reduced triphosphopyridine nucleotide (TPN). Dalgliesh (1955), however, suggested that a free-radical electron acceptor of the dehydroascorbic acid type (which is probably concerned in TPN breakdown) may itself be the active oxidizing agent.

In studies of steroid metabolism by fungi it has been shown by Dulaney, Stapley & Hlavac (1955) that zinc is a factor necessary for the 6β-hydroxylation of progesterone by *A. ochraceus* Wilhelm. In the present work with *A. niger*, it is significant that in a preliminary experiment the addition of sodium

diethyldithiocarbamate (0·001 M) markedly inhibited the hydroxylation of 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids. There is also some evidence that hydroxylation is associated with sporulation and the production of melanin by the fungus. Thus, in an experiment where the fungus was grown in submerged culture, no sporulation or pigmentation occurred and no hydroxy acids could be detected. Similar results were obtained when growth of *A. niger* in surface cultures was poor, and also with cultures of *S. laxa* which were not pigmented.

An extension of this investigation to other fungi will be reported later.

SUMMARY

1. By means of a replacement-culture technique 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids were hydroxylated in the 6-position by *Aspergillus niger*.

2. γ-(2-Naphthyloxy)-*n*-butyric acid was similarly converted into γ-(6-hydroxy-2-naphthyloxy)-*n*-butyric acid, which was subsequently β-oxidized to (6-hydroxy-2-naphthyloxy)acetic acid.

3. Higher members of the ω-(2-naphthyloxy)-*n*-alkylcarboxylic acid series ($C_{10}H_7.O.[CH_2]_n.CO_2H$, where $n = 4$–9), likewise underwent hydroxylation and β-oxidation.

4. There was no evidence to suggest why β-(6-hydroxy-2-naphthyloxy)propionic acid did not undergo β-oxidation.

5. Hydroxylation of the acetic and propionic acids led to a decrease in toxicity to the fungus.

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Some Effects of Synthetic Fluoro Compounds on the Metabolism of Acetate and Citrate

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The work described here was started to extend knowledge of the relative effects of fluoro compounds with special reference to fluoroacetate and fluorocitrate; it was hoped that it would have particular application to effects upon the liver and to the biochemical mechanism of convulsions induced by fluorocitrate.

Bartlett & Barron (1947) showed originally that acetate accumulates after fluoroacetate poisoning; but this effect is probably indirect, as Buffa & Peters (1949) found that the poisoning induced a citrate accumulation, and Peters, Wakelin, Buffa & Thomas (1953) demonstrated that this was due to the synthesis from the fluoroacetate of fluorocitrate, which blocked the tricarboxylic acid cycle at the aconitase stage. The specificity of this inhibition was clearly demonstrated on highly purified aconitase preparations by Morrison & Peters (1954). This work left questions still to be answered. For instance, the livers of male rats showed no increase in citric acid concentrations as a result of fluoroacetate poisoning, according to Buffa & Peters (1949), Potter & Busch (1950) and Fawaz & Fawaz (1953); but Ord & Stocken (1953) recently reported an apparent conversion of fluoroacetate into fluorocitrate by rat liver. There was also the problem why Hastings, Peters & Wakelin (1953) found no indication that convulsions induced by fluorocitrate were connected with any ionic imbalance.

[^{14}C]Acetate was used *in vivo* to study inhibition of acetate metabolism as a whole. Previous work by Gould, Sinex, Rosenberg, Solomon & Hastings (1949) had shown that [*carboxy*-^{14}C]acetate upon injection into rats was about 85% converted into labelled carbon dioxide in 4 hr., and Lifson & Swanson (1953) have indicated that in fluoroacetate-poisoned mice the rate of conversion of [^{13}C]acetate into radioactive carbon dioxide was decreased by approximately 45%. These results were confirmed by us and extended to fluorocitrate and fluoropyruvate. The latter compound proved particularly interesting because of its divergent action from fluoroacetate, both *in vivo* and *in vitro*.

A preliminary account of this work has appeared (Gal, Peters & Wakelin, 1954).

METHODS

Animals. Wistar-strain male rats (body weight, 70–300 g. except in rats used for experiments of Table 4) were used except for the isotopic studies when male rats of 45–55 g. were used exclusively. The animals, unless otherwise indicated, were fed upon a stock diet of rat cubes with water *ad lib.* In the experiments *in vivo*, the animals were killed by rapid immersion in liquid air. Organs were removed, weighed and worked up according to the requirements of the individual method. In the *in vitro* experiments pigeons and guinea pigs were also used. The rats for investigation of urinary-ketone substances were standardized over a period of 4 weeks before the actual experiment started; they received 20 g. of pellets daily at 4 p.m., and were weighed every 3 days. Every morning of the fifth week they were transferred to metabolic cages; the urine was collected between 9 a.m. and 4 p.m., and tested for ketone bodies by Rothera's test. In the sixth week they were divided into groups of three and injected with either fluoroacetate or fluorocitrate. Their collected urine was used for Rothera's test.

Citrate. This was determined by the method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949).

Pyruvate. This was estimated by the method of Friedemann & Haugen (1943), with 25 μg. of purified pyruvic acid as a standard. The blood samples (2 ml.) were withdrawn with heparinized needles from the hearts of unanaesthetized animals.

In the experiments with 2-^{14}C-labelled sodium acetate (Radiochemical Centre, Amersham), the animals first received an intraperitoneal injection of the fluoro compounds and then 40 min. later 30 μc/kg. of acetate (about 2·5 mg./kg.), an amount which was within the range of that tested in the experiments of Gould *et al.* (1949). The animals were then placed in an all-glass metabolic apparatus for collection of CO_2; this was a version of the apparatus of Mackenzie *et al.* (1949), modified to permit the collection of urine. The flow meter was discarded, the rate being set at about 70 bubbles/min. and regulated with a fine high-vacuum stopcock. The solutions and traps were as used by Mackenzie *et al.* (1949). The collection and preparation of $BaCO_3$ was carried out as described by Popják (1950) with 2 cm.^{2} polyethylene disks (Bettix Ltd., Arrow Works, Elm Grove, London). The radioactive measurement of the $BaCO_3$ was carried out in samples

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of 'infinite thickness', all samples being counted in duplicate. The brain, kidney, heart and liver were worked up separately, as recommended by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). The rest of the organs were pooled with the carcass. The urine samples, whenever they could be collected, were also assayed, after neutralization with NaOH to about pH 7·2 and evaporation in a desiccator *in vacuo*. The residue was taken up in 0·5 ml. of water, plated and dried under a heating lamp.

Measurement of radioactivity. All measurements were carried out on a Dynatron scaler unit with a thin-end-window Geiger–Müller counter. The background varied between 12 and 18 counts/min., and was corrected for standard deviation. All samples of counts 20 times the background were counted for 30 min., recounted, and corrected for the background and total deviation. The background counts were measured for 30–45 min. at the beginning, the middle and the end of the course of the radioactive assays. The results are given ± S.D.

Chemicals. All reagents were AnalaR grade. The fluoro compounds were of analytical purity. Sodium fluoroacetate was purchased, and fluoroacetamide was given by Dr E. D. Bergmann through Dr S. Gitter. Sodium fluorocitrate was kindly given by Dr D. E. A. Rivett or synthesized according to his method (Rivett, 1953). Enzymic fluorocitrate was prepared in the laboratory by the method of Peters *et al.* (1953). Sodium fluoropyruvate was prepared from a solution of pure fluoropyruvic acid (kindly given by Mr R. Brown) by addition of an equivalent amount of 0·1 N-NaOH. Fluoropyruvic acid was also synthesized by us by a slight modification of the method of Mager & Blank (1954) and purified by microsublimation to m.p. 82°. Diethyl fluoro-oxaloacetate was prepared according to Rivett (1953). Dimethyl fluoromalate and fluoromalamide were synthesized by Taylor & Kent (1954) and were generously put at our disposal by them. Fluoroacetylhydroxamic acid was prepared by us through the reaction of methanolic hydroxylamine in ethyl fluoroacetate. The white solid, after its recrystallization from ethyl acetate, had m.p. 79°. It was also prepared by the Hoffmann (1889) method from the corresponding amide. Phosphate buffer consisted in all cases of KH_2PO_4 neutralized to the pH stated with NaOH.

RESULTS

Experiments with [^{14}C]*acetate*

The average individual values calculated as percentage total activity of the recovered expiratory CO_2 are plotted in Fig. 1. About 50 % of the activity in the acetate control animals appears in CO_2 in about 30 min. This is somewhat faster than was observed by Gould *et al.* (1949), but it agrees with the observations of Coniglio, Anderson & Robinson (1952) on the effect of [^{14}C]acetate in fasted adult rats. All fluoro compounds injected were inhibitory; whereas in the control 75 % of the acetate was excreted as CO_2 in 4 hr., with fluoroacetate it was 40, with fluorocitrate 30 and with fluoropyruvate 50 %. It should be noted that since the acetate is injected 40 min. after the administration of the fluoro compound, considerable conversion of fluoroacetate into fluorocitrate will have already taken place in tissues where it can occur.

At first glance the amount of sodium fluorocitrate injected seems to be unusually large, especially if it is assumed that each molecule of fluoroacetate is converted into fluorocitrate. The dose of fluoroacetate injected was 0·071 m-mole/kg. and that of fluorocitrate (synthetic) was 0·145 m-mole/kg. In enzymic tests the synthetic fluorocitrate has about half the activity of the enzymic owing to the presence of unnatural isomers, so that the effective doses of fluoroacetate and fluorocitrate in these experiments were approximately equal. This raises some points for discussion.

To get some idea of the distribution of the residual activity in some parts of the body, after injection of [^{14}C]acetate, the percentage distribution was done (Table 1). The only observation of interest is that there is a trend towards increased residual activity in the carcasses of the poisoned animals. It is not possible to say whether there is some interference with acetate utilization, as suggested by Bartlett & Barron (1947), or whether in the presence of blocked aconitase there is increased conversion into other metabolites. To solve this, it would need an injection of acetate of at least 60 times the radioactivity to permit of isolation of the components for radioactive assays.

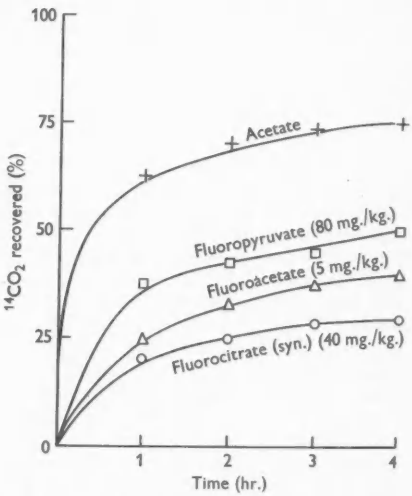

Fig. 1. Radioactivity of expiratory CO_2 from [2-^{14}C]acetate from poisoned rats (male). Percentage $^{14}CO_2$ recovered. Fluoro compounds were injected 40 min. before the injection of 30 mg. of acetate/kg. The points are the averages of pooled collections from six animals for each curve, with the exception of the fluoroacetate curve where one animal died after 3 hr. and was discarded.

Table 1. *Distribution of radioactivity after injection of* $^{14}CH_3.CO_2Na$ (30 μc/*kg.*) *into normal and poisoned rats*

Average weight of rats 50±5 g. Animals were killed at 4 hr. Doses of fluoro compounds as for Fig. 1.

	Acetate alone		Fluoroacetate and acetate		Fluoropyruvate and acetate		Fluorocitrate and acetate	
	(counts/min./g.)	(%)	(counts/min./g.)	(%)	(counts/min./g.)	(%)	(counts/min./g.)	(%)
Brain	382±39	0·41	645±132	0·70	675±155	0·75	475±187	0·53
Heart	555±33	0·60	763±164	0·82	768±285	0·85	769±196	0·85
Kidney	832±146	0·90	906±91	1·04	582±34	0·64	1 098±111	1·22
Liver	958±833	1·06	2 080±735	2·24	1 087±182	1·25	706±276	0·78
Carcass	17 000±2566	18·5	28 350±1960	30·05	22 290±1445	24·50	33 957±2380	38·00
Urine	1 840±378	2·12	803±143	0·93	776±91	0·89	7 828±892	8·92
No. of determinations	(6)		(5)		(6)		(6)	

Table 2. *Effect of intraperitoneal injections of fluoroacetate and of fluorocitrate (synthetic) on the accumulation of citrate in male rats*

Average weight of rats 50±5 g. Results are expressed as μg. of citrate/g. of wet tissue. The normal values for brain, kidney and liver are given in Table 4. Those for spleen and heart were found by Buffa & Peters (1949) to be 14 and 25 μg./g. respectively.

(*a*) *Upon accumulation of citrate in 2 hr.*

Tissue	Fluoroacetate 5 mg./kg.	Fluorocitrate 20 mg./kg.	Fluorocitrate 80 mg./kg.
Brain	178±7	151±27	364±34
Heart	798±112	108±14	365±50
Kidney	1029±40	1230±130	3060±567
Liver	33·5±5	812±89	1720±205
Spleen	596±71	510±68	635±84
No. of animals	(2)	(4)	(4)
Convulsion	+	−	+

(*b*) *Upon the rate of accumulation of citrate*

No. of animals in parentheses.

Tissue	Time (hr.)	Fluoroacetate (5 mg./kg.)	Fluorocitrate (40 mg./kg.)
Brain	1	172±8 (2)	80·7±7·3 (3)
	2	182·6±83 (3)	221·5±58·5 (4)
	3	200±24·8 (4)	233±25·6 (4)
	4	205±25·3 (4)	248·5±36·5 (4)
Kidney	1	597·6±93 (3)	1508±102 (4)
	2	1110±83 (4)	2329±153·9 (6)
	3	1499±302 (4)	2725±423 (4)
	4	1594±751 (4)	2456±251 (4)
Liver	1	30·6±2·6 (3)	956±44·6 (3)
	2	50·5±17 (4)	1313±179 (6)
	3	34·9±1·9 (4)	1450±126 (3)
	4	44·4±5·7 (4)	1496±236·5 (4)

Accumulation of citrate

Table 2*a* shows the average content of citric acid in the same strain of rats in various organs 2 hr. after poisoning by fluoroacetate and fluorocitrate. In fourteen out of fifteen cases, fluoroacetate induced no increase in the citrate concentration of the liver. It should be emphasized that these were male rats, as the citrate values are known to be different in female animals (Dubois, Cochran & Zerwic, 1951).

The picture for fluoroacetate at 1 hr. after injection confirms that of Buffa & Peters (1949); see also Potter & Busch (1950). The injection of fluorocitrate, however, even at 20 mg./kg., produced a surprising difference in the liver, where the citrate content increased to 24 times the normal. This was also shown by the triethyl ester of fluorocitric acid.

In Table 2*b*, the relative rates of citrate accumulation have been determined. It is to be noted

particularly that in both the liver and the kidney, but especially in the liver, there is an immediate rise of citric acid following fluorocitrate injection, suggesting that it produces its action rapidly. Taken together, the interpretation appears to be that fluorocitrate penetrates the liver cells directly and so blocks the tricarboxylic acid cycle, whereas fluoroacetate, even if it penetrates, is not synthesized to fluorocitrate. This latter behaviour would be consistent with the failure to find that fluorocitric acid is formed from fluoroacetate and oxaloacetate by pigeon-liver particles *in vitro* (Peters & Wakelin, 1952). In experiments *in vitro*, fluorocitrate caused large accumulations of citric acid in liver homogenates.

The results with brain were somewhat equivocal. There was a quicker rise in citric acid with fluoroacetate than with fluorocitrate; but this difference is not statistically significant. Fluoroacetate gave convulsions in 30 min., whereas with fluorocitrate the animals only convulsed with a dose of 80 mg./kg.; 40 mg./kg. did not give convulsions, nor could its action be potentiated by simultaneous administration of urea to increase penetration, or by injection of 0·8 mg. of fluoroacetate/kg.

In order to make it clear that in the rat also the intracerebral injection of fluorocitrate produces effects not given by fluoroacetate, some intracerebral injections were made in young rats with minimal doses. The previous comparison had been made on pigeons (Peters & Wakelin, 1953), and the experiments of Dawson & Peters (1955) made for another object had been done with amounts too large to determine this point. Intracerebral injections of 1·5 μg. of fluorocitrate (enzymic) were convulsive in six animals of weight 48–55 g., whereas 10 μg. of fluoroacetate did nothing, except in one animal out of six (the first tried, which convulsed after 55 min., but did not die). It may be reckoned that for these animals approximately 1·2 μg. of fluorocitric acid was the limiting amount needed for producing convulsions. Table 3 gives the distribution of citric acid in brain, kidneys and the liver at death following such intracerebral injection; some of the injected fluorocitrate occasionally appears to get from the brain into the kidney, where it induces a rise of citric acid. A tendency for a similar phenomenon can be seen in the results of Dawson & Peters (1955), although their experiments were done with much larger amounts of fluorocitrate.

The experiments made *in vivo* give support to the *in vitro* experiments which have indicated that there is some difference in the pathway for the conversion of fluoroacetate and acetate into fluorocitric and citric acids respectively. This can be judged from the fact that, like fluorocitric acid, fluoroacetate cuts down the conversion of [^{14}C]acetate into CO_2, and yet is clearly not activated by liver. In addition,

Table 3. *Effect of intracerebral injection of fluorocitrate (enzymic) on citrate accumulation*

Average weight of rats 50 ± 1·5 g.

Fluorocitrate injected (μg.) (as acid)	5·3	5·3	8
First convulsion (min.)	36	33	37
Killed (min.)	56	71	78
	Citrate (μg./g. of wet tissue)		
Brain	170	177	142
Kidney	28·4	87·5	165
Liver	11·3	5·6	8·5

we have noticed that the urine of animals treated with fluorocitrate gave a positive Rothera reaction for acetoacetic acid. Quantitative assays are in progress by the method of Greenberg & Lester (1944). There may be a metabolic diversion of active acetyl fragments with fluorocitrate, presumably in the liver; this does not occur with fluoroacetate. One of us (Peters, 1955) has concluded that there must be two paths for acetate metabolism, only one of which is used in fluoroacetic acid activation. It seems certain now that fluoroacetyl-coenzyme A (CoA) must be a component of the path for fluoroacetate, as this is implied by the experiments of Brady (1955) and of Elliott & Marcus (1955), who synthesized fluoroacetyl-CoA and showed that it gave fluorocitrate in the presence of oxaloacetate and condensing enzyme. Some difference was found, however, in the chemical nature, reactivity and resistance to hydrolysis between the acetyl- and the fluoroacetyl-CoA. To make certain that the inability to detect fluoroacetyl-CoA in enzymic tests was not due to failure of fluoroacetylhydroxamate to give the usual colour reaction with ferric chloride, we have synthesized fluoroacetylhydroxamic acid and found that it gives the iron reaction.

We have also tried several fluoro compounds along with fluoroacetylhydroxamate to find how far they interfere with the metabolism of pyruvate and acetate; the results of the intraperitoneal injections are given in Table 4. It will be noted that the fluoroacetylhydroxamic acid produced a significant increase of citric acid only in the kidney; yet, unexpectedly, it produced convulsions which appeared 90 min. after the administration of 8 mg./kg. Much larger doses of diethyl fluoro-oxaloacetate gave the same citric picture without convulsions. Fluoromalamide produced no effect except for a small rise in kidney citrate. In all these cases it appeared that there was little if any tendency to convert any active fluoroacetyl into fluorocitrate (as judged by citrate accumulation). The most remarkable result was found with fluoropyruvate, the preliminary results of which are reported below

Table 4. *Effect of the intraperitoneal injection of various fluoro compounds and of one iodo compound on the citrate accumulation*

Compound	Average wt. of animal (g.)	Dose (mg./kg.)	Time (hr.)	Citrate (μg./g. of wet tissue) Brain	Kidney	Liver	No. of expts.
Fluoroacetylhydroxamic acid	80	8·0	4	59±17·5	251±83	12·4±3·8	6
Fluoroacetamide	100	10·0	2	120±21	247±61	20±6·9	2
Dimethyl fluoromalate	85	150·0	2	43±6	48·2±2·5	16±3·5	2
Fluoromalamide	100	300·0	2	46±5	125±33	62±5·1	2
Diethyl fluoro-oxaloacetate	50	150·0	2	41±3·8	119±21	22·5±2·5	2
	400	150·0	2	53±7	344±100	15·5±0·5	2
Iodoacetic acid	612	1·80	1	—	74·2±16	33±9·2	2
Control	578	—	—	39±6	45±7	52±12	2
	100	—	—	20±6	29±13	3·5±0·4	2

at greater length, and show that this compound is metabolized differently from fluoroacetate or the even-numbered ω-fluoro fatty acids.

Fluoropyruvic acid

It has long been thought by those working in the field of fluoro compounds (see Chenoweth, 1949) that fluoropyruvic acid should be investigated. The compound recently became available through the work of Mager & Blank (1954), whose synthesis has been confirmed by Mr R. Brown and by one of us (E.M.G.) in England.

Experiments in vivo. When injected *in vivo* fluoropyruvate produced no change at all in the citric acid levels; yet some change in the animal must have occurred because of the convulsions it could produce in 4 hr. with a dose of 80 mg./kg. It is noteworthy that whereas the animals poisoned with fluoroacetate and fluorocitrate did not recover, the animals treated with fluoropyruvic and fluoroacetylhydroxamic acids survived at the dose levels tried. At first it was thought that fluoropyruvate might interfere with pyruvate metabolism, especially in view of the enzyme results given below (and of the report of Mager & Blank, 1954). Table 5 shows, however, that there was lower blood pyruvate than normal in the animals poisoned with fluoropyruvate. The following substances were tried with negative results in each of two animals, in the hope of reversing the poisoning: glucose, calcium gluconate, thiamine-HCl and pyruvic acid. This failure of fluoropyruvic acid to affect the tricarboxylic acid cycle is all the more surprising because it would be expected that, like pyruvic acid, it would yield an active fluoroacetyl fragment which in turn would form fluorocitrate, and consequently cause accumulation of citrate.

Experiments in vitro. The methods used have been described in previous papers; the particle preparations are similar to those of Buffa, Peters & Wakelin (1951) and of Liébecq & Peters (1949).

Table 5. *Effect of the intraperitoneal injection of fluoropyruvic acid (80 mg./kg.) on citrate and blood pyruvate accumulation in 4 hr.*

Rats: 100±5 g. for citrate and 220±15 g. for pyruvate.

	No. of determinations	Citrate (μg./g. of wet tissue)
Brain	4	26·1±9·2
Heart	4	34·1±5
Kidney	4	39·8±7
Liver	4	22·5±3·1
		Blood pyruvic acid (mg./100 ml.)
Poisoned	4	0·578±0·028
Control	2	1·317±0·042

Table 6. *Effect of fluoropyruvic acid on carboxylase of yeast*

Each bottle contained 100 mg. of yeast in 1 ml. of 0·1 M phosphate buffer (pH 6·2), 0·1 ml. of $MgCl_2$ (4·0 μmoles of Mg^{2+}), 0·2 ml. of sodium pyruvate (54 μmoles) ± 0·1 ml. of neutralized fluoropyruvic acid (16·3 mM); the whole was made up to 3·2 ml. with phosphate buffer. The small residual CO_2 production was subtracted from the results. Pyruvate was added from the side tubes after equilibration. Temp. 28°.

	CO_2 evolved (μl./30 min.)	Percentage of control
Pyruvate alone	732	—
Pyruvate and fluoropyruvate	272	37·1

(i) *Pigeon-brain particles.* In the experiments on pigeon-brain particles it was decided to test for oxidation and conversion into fluorocitrate by the use of the pigeon-brain pyruvate oxidase system. As has been shown in experiments in this Laboratory, when pigeon-brain particles are supplemented with pyruvate plus fumarate and reinforced with magnesium and adenosine triphosphate (ATP), the pyruvate is readily oxidized, and in the presence of fluorocitrate there is an accumulation of citric acid. If therefore fluoropyruvate is oxidized in a similar

Table 7. *Effect of fluoropyruvate upon* (a) *the synthesis of citric acid from fumarate, and* (b) *the metabolism of citric acid by kidney particles*

All conical flasks contained as a final addition 2 ml. of kidney particles (guinea pig) suspended in 1·6 ml. of 0·134 M-KCl and 0·4 ml. of 0·5 M phosphate buffer (pH 7·2); the flask also contained 0·1 ml. of Mg^{2+} (4·0 μmoles of $MgCl_2$) and 0·1 ml. of ATP (1·2 μmole) in (*a*) 0·2 ml. of sodium fumarate (20 μmoles); (*b*) 0·4 ml. of sodium citrate (10 μmoles); 0·134 M-KCl was added to make the final volume of 3·0 ml. After shaking for 30 min. at 38·1°, the reaction was stopped with 1 ml. of 25% trichloroacetic acid, and after filtration the contents of duplicate flasks were made up to 16 ml., from which samples were taken for estimation of citric acid.

Blocking agent	Addition	Citric acid accumulated (μmoles)	Citric acid disappeared (μmoles)
Expt. 1 (*a*) None	Fumarate alone	0·16	—
	Fumarate + fluoropyruvate (6·29 mM)	0·13	—
(*b*) Malonate (0·034 M)	Citrate (10 μmoles)	—	9·51
	Citrate + fluoropyruvate (6·29 mM)	—	8·92
Expt. 2 (*a*) Fluorocitrate (20 μg., synthetic) (0·095 μmole)	Fumarate alone	7·52	—
	Fumarate + fluoropyruvate (3·14 mM)	0·76	—
	Fumarate + fluoropyruvate (6·29 mM)	0·58	—
(*b*) Malonate (0·034 M) (no fluorocitrate)	Citrate (10 μmoles)	—	9·83
	Citrate + fluoropyruvate (3·14 mM)	—	9·92
	Citrate + fluoropyruvate (6·29 mM)	—	9·93

way to pyruvate, it should lead to fluorocitrate formation, which should both block the respiration and cause an increased accumulation of citric acid. Fluoropyruvate causes a strong inhibition of pyruvate oxidation in the pigeon-brain system (Fig. 2). In the presence of 0·633 mM fluoropyruvate (0·2 mg./3 ml. in the flask) there is already an inhibition of approximately 35% in the oxygen uptake. This is not, however, associated with an accumulation of citric acid. In the legend to Fig 2, inhibitions of citric acid formation are recorded which have been found to accompany the decrease of oxygen uptake in these experiments. These are more variable and generally greater than the inhibition of oxygen uptake. It seems clear, therefore, that there must be interference with the pyruvate-dehydrogenase step in this oxidation and that there has been no synthesis of an inhibitor for the citric acid stage of the tricarboxylic acid cycle.

The inhibition of the early stage of metabolism of pyruvate has been shown again by studying the effect upon yeast decarboxylation of pyruvic acid. Table 6 shows the decrease caused by fluoropyruvate in this decarboxylation, one of three similar results with dried baker's yeast.

(ii) *Kidney particles* (*guinea pig*). The brain-particle experiments indicated that there was no synthesis of fluorocitric acid by brain tissue; instead there was a definite interference with pyruvate oxidation which was reflected in the formation of citric acid. Since brain tissue, at any rate under these conditions, does not synthesize fluorocitric acid from fluoroacetate in the presence of pyruvate and fumarate, it was thought that the failure of fluorocitric acid synthesis should be confirmed with kidney tissue. In our ordinary experiments with fumarate alone and kidney particles, sufficient active acetyl fragments are formed to make citric acid. It has been shown that in the presence of fluoroacetate and fumarate there is an extra accumulation of citric acid owing to the synthesis of fluorocitric acid (Liébecq & Peters, 1949; Peters *et al.* 1953). With fumarate there is a maximal accumulation of citrate when the preparation is blocked with fluorocitrate. On the other hand, when citrate itself is used as a substrate, fluorocitrate can be determined by its action in blocking aconitase and so preventing the disappearance of citrate. The latter has been the test consistently used in the isolation of fluorocitric acid. The experiments in Table 7 show that in kidney particles, when blocked with fluorocitrate, 3·14 mM fluoropyruvate blocked the formation of citrate by approximately 90%. On the other hand, this amount had no inhibiting effect upon citrate disappearance. In another experiment in the absence of a fluorocitrate block, fluoropyruvate induced no accumulation of citrate. Both these experiments, which have been confirmed, show that fluoropyruvate has an entirely different effect from the expectation if it had been activated to an active fluoroacetyl fragment. More work is required to decide the exact position of the block in the initial stages of pyruvate oxidation and to determine whether fluoropyruvic acid blocks other enzymes too.

In the preliminary note of these experiments it was stated that the effect was not reversible, and this failure to reverse has been found in a small number of experiments; but in further work there seemed to be some conditions under which some degree of reversibility can be observed. This matter, therefore, requires further exploration.

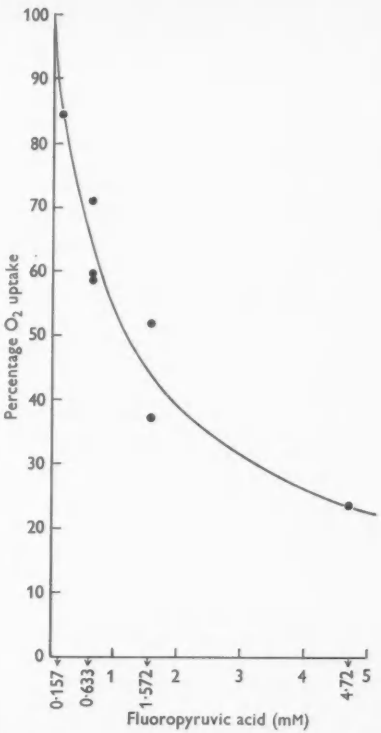

Fig. 2. Inhibition of pyruvate oxidation by fluoropyruvate in pigeon-brain particles; points at 0·633 mM with centrifuged particles, the rest with uncentrifuged particles. The total volume of 3·0 ml. contained in 1·0 ml. of fumarate (10 μmoles), pyruvate (27 μmoles), Mg^{2+} (4·0 μmoles $MgCl_2$), ATP (1·2 μmoles), KCl (0·134 M); and in the remaining 2·0 ml. of brain particles in KCl (0·134 M) and 0·1 M phosphate buffer (pH 7·2). Citric acid accumulations were also determined, and gave, as compared with the control (100%): at 0·157 mM 87%; 0·633 mM 31, 50, 53, 68, 79%; at 1·572 mM 29, 34%; at 4·72 mM 29%.

DISCUSSION

It is clear that the difference in citric acid content of the liver after injection of fluorocitric acid forms a distinguishing clinical mark for identifying the presence of fluorocitric as distinct from fluoroacetic acid.

In our studies of the rate of carbon dioxide production from acetate there was striking agreement between our values and those of Lifson & Swanson (1953). These authors gave the rate 9·35 m-moles/hr./100 g. of mouse, while ours, as shown in Fig. 1, was 9·65 m-moles/hr./100 g. of rat. With fluoroacetate their rates were 5·59 m-moles/hr./100 g. for 10 mg. of fluoroacetate/kg. and 4·60 for 20 mg./kg.; ours was 4·09 m-moles/hr./100 g. for 5 mg./kg. in the rat. We found that the rate of CO_2 production was 5·30 m-moles/hr./100 g. for 40 mg. of (synthetic) fluorocitrate/kg., while the rate of CO_2 for fluoropyruvate (8 mg./kg.) was 8·15 m-moles/hr./100 g. In assessing the close correlation it is important to remember the wide variation in LD_{50}, even for different strains of rat (Chenoweth, 1949). Both fluoroacetate and fluorocitrate were given at levels causing convulsions, but fluorocitrate was much more inhibitory to the rate of production of respiratory CO_2. Our results should not be interpreted as showing more than a trend.

The lack of citrate accumulation in the liver of male rats poisoned by fluoroacetate and its indication of failure to form fluorocitric acid suggested that there is a mechanism competing with the formation of fluoroacetyl-CoA.

SUMMARY

1. Injections into rats of some fluoro compounds followed by [2-^{14}C]acetate led to reduction in the $^{14}CO_2$ expired as follows: fluoroacetate 40%, fluorocitrate 30%, and fluoropyruvate 50%. The carcasses of the poisoned animals contained more radioactivity than the other tissues examined.

2. In male rats, whereas generally fluoroacetate induced no rise in citric acid content of the liver, fluorocitrate injections caused immediate rise in the citric acid level.

3. Fluorocitrate when injected intracerebrally has a high toxicity for the rat, as has been shown already in pigeons and rabbits.

4. The urine from animals injected with fluorocitrate gave a marked Rothera's test.

5. Fluoroacetylhydroxamic acid when injected gave convulsions in 90 min. with only slight increases in kidney citrate. Diethyl fluoro-oxaloacetate also increased citrate in kidney, but without convulsions. Fluoromalamide gave no effect.

6. Fluoropyruvic acid when injected gave convulsions in 4 hr., with no increase in citric acid. Several substances tried did not reverse the effect. *In vitro*, fluoropyruvic acid inhibited the metabolism of pyruvic acid.

One of us (E.M.G.) gratefully acknowledges the support of the National Foundation for Infantile Paralysis, Inc. (U.S.A.), throughout the entire course of these experiments. He also wishes to thank Professor Wendel H. Griffith of the Department of Physiological Chemistry, Medical Centre, University of California, Los Angeles, for the use of the departmental facilities for some of the experiments. The valuable help of Drs P. Buffa, P. W. Kent, L. A. Stocken, and particularly that of Mr N. F. Taylor, is greatly appreciated. Our thanks are also due to Mr H. Vincent for the skilled construction of the metabolism apparatus and other

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Indirect Analysis of Corticosteroids

3. THE DETERMINATION OF STEROIDAL DIHYDROXYACETONES*

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Earlier papers of this series (Appleby, Gibson, Norymberski & Stubbs, 1955; Appleby & Norymberski, 1955) introduced two analytical methods, each of which involved a sequence of specific reactions designed to transform an accurately defined group of corticosteroids into 17-oxosteroids and the measurement of the latter as chromogens in the Zimmermann reaction. The same general principle has now been employed to develop a further method of steroid analysis. It is based on the observation (Norymberski, 1956) that steroidal dihydroxyacetones (17α:21-dihydroxy-20-oxopregnanes) undergo hydrogenolysis at the $C_{(17)}$–O bond when treated with zinc powder in boiling aqueous acetic acid; so, for instance, cortisone (I) was converted into 11-dehydrocorticosterone (II). Consequent upon the removal of the 17-hydroxyl group the dihydroxyacetones lose their 17-ketogenic property (convertibility into 17-oxosteroids on oxidation with bismuthate; see Brooks & Norymberski, 1953). On the other hand, it was expected that 17:20-diols and 17:20:21-triols, i.e. the two other structural types of the 17-ketogenic group, will resist the action of zinc and hence that it should be possible to measure them selectively as 'zinc-resistant 17-ketogenic steroids'. The proposed analytical procedure was briefly outlined in a preliminary communication (Norymberski & Stubbs, 1955).

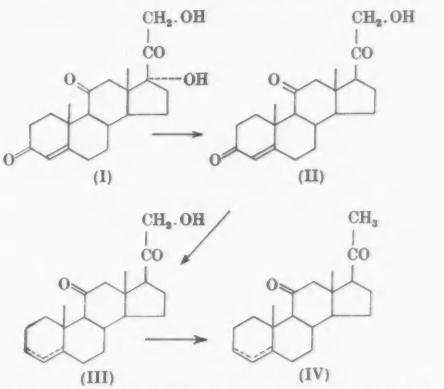
The preparative experiments referred to above were performed with only a moderate excess of zinc (10:1, w/w). When cortisone was reduced under conditions considered more suitable for analytical purposes, wherein a much larger excess of zinc (10 000:1, w/w) was employed, it was found that after 1 hr. reduction with zinc, and following oxidation with bismuthate, the amount of Zimmermann chromogens was decreased to a constant level. The nature of the residual chromogenic contribution, whose magnitude corresponded to about 20 % of the quantity expected if the cortisone had not been

* Part 2: Appleby & Norymberski (1955).

affected, was not immediately apparent. It could not derive from unchanged cortisone since the reduction product gave no colour with phenylhydrazine in sulphuric acid (Porter & Silber, 1950). Neither could it derive from 11-dehydrocorticosterone (II), since this compound would have been converted by bismuthate into an aetio-acid and the latter removed by the alkali wash preceding the Zimmermann reaction. It was therefore suspected that under the reaction conditions employed cortisone underwent more profound structural changes than in the preparative experiments. This view was confirmed by experiments in which cortisone was treated with various amounts of zinc (100-fold to 10 000-fold, w/w): it was found that (i) at all ratios of the reactants the reaction products were practically non-chromogenic in the Porter–Silber test, (ii) the extinction at 238 mμ. fell rapidly with increasing amounts of zinc, and (iii) the amount of formaldehyde released on oxidation with bismuthate fell slowly with increasing amounts of zinc (see Fig. 1). These results are interpreted on the assumption that the products formed at low ratios of reactants are intermediates in the reactions occurring at higher ratios. The initial step is then the removal of the 17-hydroxyl group, since it occurs with a tenfold excess of the reagent; the results of the Porter–Silber test are therefore taken as an indication of the extent of the reaction at $C_{(17)}$. The disappearance of specific absorption at 238 mμ. obviously indicates the reduction of the 4-en-3-one system; the reduction product gave a yellow colour with tetranitromethane and a negative test with trichloroacetic acid (Rosenheim, 1929), it must therefore contain an olefinic bond but no hydroxyl group in the allylic position. Finally, the formaldehydogenic property is considered to be associated with the presence of the 21-hydroxyl group.

On the basis of these observations the reaction of cortisone with a large excess of zinc is tentatively formulated as follows. Reductive removal of the 17-hydroxyl group converts cortisone (I) into 11-dehydrocorticosterone (II); this is followed by reduction of the 4-en-3-one grouping, leading to a 21-hydroxypregnene-11:20-dione (III), and finally removal of the 21-hydroxyl gives a pregnene-11:20-dione (IV). The properties expected from compounds (I)–(IV) in the analytical tests employed (see Table 1) provide a satisfactory rationalization of the experimental findings; in particular, the formulation of the end product as a 20-ketone unsubstituted at $C_{(21)}$ explains the residual chromogenic contribution to the Zimmermann reaction.

Further confirmation of the proposed reaction sequence was obtained by treating tetrahydrocortisone (V) with a 500-fold excess of zinc. After acetylation three compounds were isolated: 3α:21-diacetoxypregnane-11:20-dione (VI*b*), which was the



Dotted lines in formulae (III) and (IV) denote two likely positions of the double bond.

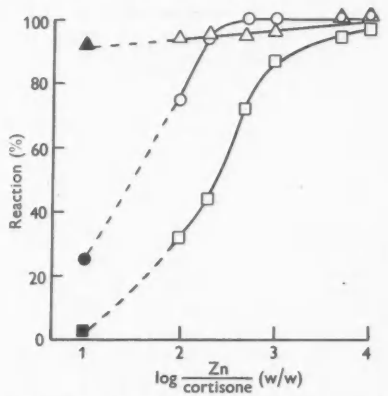


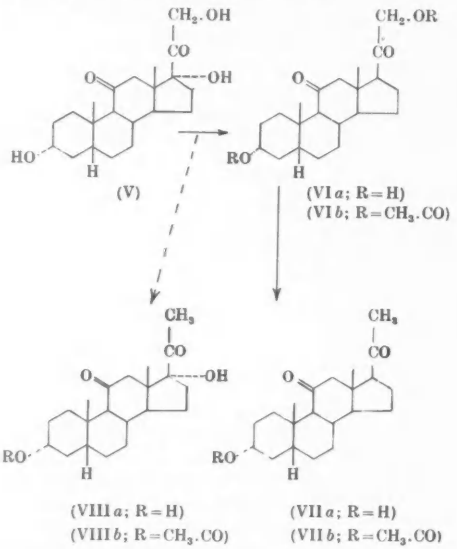
Fig. 1. Reaction of cortisone with zinc at various ratios of the reactants. ○, Reaction at $C_{(3)}$ based on extinction at 238 mμ.; △, reaction at $C_{(17)}$ based on results of the Porter–Silber reaction; □, reaction at $C_{(21)}$ based on release of formaldehyde with bismuthate. Filled-in points are approximate values derived from several preparative experiments.

Table 1. *Summary of properties of cortisone (I) and its transformation products after reduction with zinc*

Test	Compound I	II	III	IV
Porter–Silber reaction	+	−	−	−
17-Ketogenic property	+	−	−	(±)*
Specific absorption at 238 mμ.	+	+	−	−
Release of formaldehyde	+	+	+	−
Tetranitromethane	−	−	+	+

* Weakly chromogenic in the Zimmermann reaction and resistant to bismuthate.

sole reaction product when a tenfold excess of zinc was employed (Norymberski, 1956), 3α-acetoxypregnane-11:20-dione (VII*b*), and a small quantity (approx. 5 %) of 3α-acetoxy-17α-hydroxypregnane-11:20-dione (VIII*b*). To establish whether 3α:17α-dihydroxypregnane-11:20-dione (VIII*a*) is the end product of a side reaction or an intermediate capable of further reaction with zinc, the compound was treated with a 10000-fold excess of the reagent, and the Zimmermann chromogens were measured (i) without further treatment, (ii) after treatment with bismuthate, and (iii) after treatment with borohydride and then with bismuthate. In each case the colorimetric result was practically identical with that found in a corresponding experiment in which the treatment with zinc was omitted, which strongly indicates that the 17:20-ketol grouping of the model compound remained essentially unchanged during treatment with zinc.



In Table 2 evidence is presented in support of the expected resistance to zinc of 17:20-diols, 17:20:21-triols and 17-ketones. The table also summarizes the properties of dihydroxyacetones and 17:20-ketols already discussed and, finally, includes some observations, in the present context of only incidental interest, on the reaction of 21-acetoxy-17α-hydroxy-20-ones with zinc.

The information so far obtained seemed sufficient to apply the zinc treatment to urines. In an exploratory experiment urine samples were diluted with an equal volume of acetic acid and boiled with zinc (0·1 g./ml.) for various times: the 17-oxosteroids remained unaffected, even after 3 hr. of this treatment, and the 17-ketogenic steroids were depleted to a constant value reached after about 1 hr. Treatment with zinc for 1 hr. was adopted as the standard procedure and employed in all subsequent experiments.

Addition of various amounts of cortisone (10–50 μg./ml.) to samples of one urine specimen assayed by the standard procedure gave rise to an increase of Zimmermann chromogens corresponding to a recovery of 7–10 % of cortisone (in terms of androst-4-ene-3:11:17-trione). When a constant amount of cortisone (10 μg./ml.) was added to samples of thirty-four different urine specimens the observed chromogenic increments corresponded to an average recovery of 6 ± 11 % of cortisone. On the other hand, 3α:17α:20β-trihydroxypregnan-11-one and 17α:20β:21-trihydroxypregn-4-en-3-one contributed to the assay to the extent expected from their conversion into 17-oxosteroids. Finally it was found that urines which were treated with borohydride (cf. Appleby *et al.* 1955) gave identical chromogenic values after (i) treatment with bismuthate, and (ii) treatment with zinc followed by treatment with bismuthate. Since borohydride reduces all urinary ketones, including dihydroxyacetones, to the corresponding alcohols, the above finding clearly demonstrates the specificity of action of zinc within the group of native urinary 17-hydroxycorticosteroids.

Some results of the zinc–bismuthate method are summarized in Fig. 2, where the daily excretion levels of steroidal dihydroxyacetones (SDHA) are plotted against those of 17-ketogenic steroids (17-KGS) for a group of subjects comprising (i) normals (nine men, twelve women), (ii) seven women in the last trimester of pregnancy, (iii) fifteen rheumatoid-arthritic patients receiving

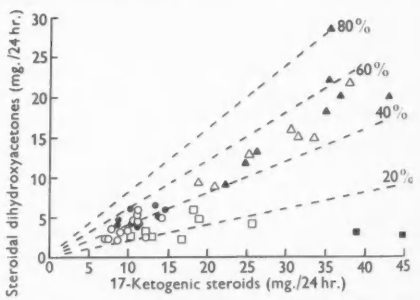


Fig. 2. Composition of 17-ketogenic steroids in human urine. ○, Normal females; ●, normal males; □, last trimester of pregnancy; ■, adrenogenital syndrome; △, rheumatoid arthritics receiving cortisone acetate; ▲, rheumatoid arthritics receiving corticotrophin. Interrupted lines are boundaries of the indicated percentage content of dihydroxyacetones.

Table 2. *Summary of reactions of model compounds with zinc**

Side chain	Compound	Zinc/steroid (w/w)	Side chain of product	Evidence
	Cortisone, Cortisol, Tetrahydrocortisone	10:1	>CH.CO.CH_2.OH	Isolation of products†
CH_2.OH, CO, C..OH	Tetrahydrocortisone	500:1	>CH.CO.CH_2.OH; >CH.CO.CH_3; >C(OH).CO.CH_3	Isolation of products‡
	Cortisone	10 000:1	>CH.CO.CH_3; [>C(OH).CO.CH_3 (?)]	Decreased value as 17-KGS; negative Porter–Silber test; not formaldehydogenic‡
CH_2, CO, C..OH	3α:17α-Dihydroxypregnane-11:20-dione	10:1	Unchanged	Isolation of starting material†
		10 000:1	Unchanged	No significant change in assays of 17-KS, 17-KGS, and 17-OH CS‡
CH_2.OH, CH.OH, C..OH	17α:20β:21-Trihydroxypregn-4-en-3-one	10 000:1	Unchanged	17-Ketogenic property retained‡
CH_3, CH.OH, C..OH	3α:17α:20β-Trihydroxypregnan-11-one	10 000:1	Unchanged	17-Ketogenic property retained‡
O, C	Dehydro*epi*androsterone	10 000:1	Unchanged	Chromogenic value in the Zimmermann reaction retained‡
CH_2.OAc, CO, C..OH	Cortisone acetate	10:1	Unchanged	Isolation of starting material†
		10 000:1	>C(OH).CO.CH_3 (?)	Negative Porter–Silber test‡
	Tetrahydrocortisone acetate	10 000:1		Increased value as 17-KS; no significant change as 17-KGS and 17-OH CS‡

* See also Table 4. † Norymberski (1956). ‡ This paper.

cortisone acetate (by mouth) or a preparation of the corticotrophic hormone (by intramuscular injection), and (iv) two cases of the adrenogenital syndrome. Within the normal group the ratio of SDHA to 17-KGS varied between 20 and 60 %. At higher excretion levels caused by the administration of cortisone acetate or corticotrophin the range of the same ratio was raised to 40–80 %, in pregnancy it was lowered to 10–30 %, and in the adrenogenital syndrome the ratio was less than 10 %.

The average normal excretion values of SDHA were (in mg.-equiv. of dehydro*epi*androsterone/24 hr.): 4·9 (range 4·0–6·4) for men, and 3·6 (range 2·0–5·8) for women. Sandberg, Nelson, Glenn, Tyler & Samuels (1953) found for glucuronide-bound dihydroxyacetones, measured as Porter–Silber chromogens (Glenn & Samuels, 1953), the following average normal excretion values (in mg.-equiv. of cortisol/24 hr.): 5·3 (range 3·3–9·3) for men and 3·2 (range 2·1–5·8) for women. In view of the profoundly different techniques employed in the two analytical methods, agreement between the results must be treated with caution.

EXPERIMENTAL

Melting points were determined on a Kofler stage. Rotations were measured in $CHCl_3$ (at 15–20°) and ultraviolet absorption spectra in ethanol. Analysis was by Weiler and

Strauss (Oxford). The Zimmermann reaction was performed according to Zygmuntowicz, Wood, Christo & Talbot (1951) and colorimetric readings were taken at 430 and 520 mμ. and corrected (Medical Research Council, 1951). Chromogens with phenylhydrazine and sulphuric acid were determined essentially by the method of Porter & Silber (1950), the reaction being performed overnight at room temperature and the extinction measured at 430 mμ. Formaldehydogenic steroids were determined by the method of Brooks & Norymberski (1953). Reductions with borohydride were carried out as previously described (Appleby *et al.* 1955), except that potassium borohydride was used instead of the sodium salt.

Preparative treatment of tetrahydrocortisone (V) with zinc powder

Tetrahydrocortisone (100 mg.) in aqueous acetic acid (50%, v/v; 100 ml.) was treated with Zn powder (50 g.; AnalaR) for 1·5 hr. at reflux. After cooling, the Zn was filtered off, refluxed with ethanol and filtered off again. Ice and then 3N-NaOH (200 ml.) were added and the mixture was extracted with ether in the usual manner. The crude product (80 mg.) was acetylated with acetic anhydride in pyridine at room temperature. The product was chromatographed on neutral alumina (8 g.) by a gradient-elution technique (see Appleby & Norymberski, 1955) with the solvent systems benzene–light petroleum (1:1)/benzene/benzene–ethyl acetate (98:2); sixty 4 ml. fractions were collected.

Fractions 6–9 (m.p. range 110–131°; 21·5 mg.) gave from acetone–hexane heavy prisms, m.p. 130–131°, $[\alpha]_D$ +131° (*c*, 1·25). (Found: C, 73·8; H, 9·45. Calc. for $C_{23}H_{34}O_4$: C, 73·8; H, 9·15%.) The compound was identified as 3α-acetoxypregnane-11:20-dione (VII*b*) by its unchanged m.p. on admixture with an authentic specimen. Euw, Lardon & Reichstein (1944) reported for the compound m.p. 132–133°, $[\alpha]_D$ +122° (in acetone).

Fractions 13–25 (m.p. range 100–110°; 29·5 mg.) gave from acetone–hexane small plates, m.p. 107–109°, $[\alpha]_D$ +123° (*c*, 1·40). The mixed m.p. with authentic 3α:21-diacetoxypregnane-11:20-dione (VI*b*; Norymberski, 1956) was unchanged.

Fractions 39–43 (m.p. range 193–202°; 6 mg.) gave from acetone–hexane prisms, m.p. 203–206°. The compound was identified as 3α-acetoxy-17α-hydroxypregnane-11:20-dione (VIII*b*) by comparison of m.p. and infrared spectrum with those of an authentic specimen.

ANALYTICAL

Symbols and definitions

17-*KS*. 17-Oxosteroids in terms of dehydro*epi*androsterone.

Total 17-*KS*. 17-KS found after treatment with bismuthate.

Zn/*total* 17-*KS*. 17-KS found after treatment with Zn and then with bismuthate.

17-*KGS* (17-*ketogenic steroids*). Total 17-KS − 17-KS.

Zn/17-*KGS* (*zinc-resistant* 17-*KGS*). Zn/total 17-KS − 17-KS.

SDHA (*steroidal dihydroxyacetones*). 17-KGS–Zn/17-KGS = total 17-KS − Zn/total 17-KS.

General procedure for treatment with zinc

A solution of the analytical sample (0·1–0·3 mg., unless otherwise stated) in aqueous acetic acid (50%, v/v; 10·0 ml.) is refluxed with Zn powder (1·0 g.; AnalaR) for 1 hr. (The reaction is conveniently performed in test tubes (6 in. × ¾ in.) fitted with cold-finger condensers and placed in an aluminium heating block maintained at 120–140°.) The reaction mixture is rapidly cooled to room temperature and further treated in one of the two following ways.

(i) *For the determination of* Zn-*resistant total* 17-*KS*. A sample (4·0 ml.) of the supernatant is shaken with $NaBiO_3$ (0·5 g.; AnalaR) for 0·5 hr. and worked up by reducing surplus reagent with $Na_2S_2O_5$ and extracting in the usual manner (see Gibson & Norymberski, 1954; Appleby *et al.* 1955). Samples of the washed extract of suitable size are taken for the Zimmermann reaction.

(ii) *For other analytical tests*. A sample (8·0 ml.) of the supernatant is treated with 3N-NaOH (4·0 ml.) and then extracted with ethylene dichloride (25·0 ml.); the extract is washed successively with water (5 ml.) and 3N-NaOH (5 ml.) and filtered through paper. Suitable portions of the filtrate are taken for the tests employed.

Variation of reaction time. Samples (100 μg. each) of cortisone were treated by the general procedure (i) except that the time of reaction with Zn was varied between 10 and 90 min. In control experiments addition of Zn was omitted. Each experiment was duplicated. The results (mean values) are in Table 3.

Variation of reactant ratio. Samples of cortisone (0·1–10·0 mg.) were treated by the general procedure (ii). Samples of the final extract, equivalent to 22·4–64·0 μg. of starting material, were taken for the measurement of (*a*) the extinction at 238 mμ., (*b*) Porter–Silber chromogens, and (*c*) formaldehyde, released by bismuthate, as chromogen with chromotropic acid. Each measurement was corrected by subtraction of a value found for the corresponding process blank (without addition of cortisone). The results are presented in Fig. 1.

Tetranitromethane gave a yellow colour with products obtained at all concentrations of cortisone; no colour was produced with trichloroacetic acid in $CHCl_3$.

Variation of substrate. Samples of steroids listed in Table 4 were treated by the general procedure (i) or (ii),

Table 3. *Effect of zinc treatment on the 17-ketogenic property of cortisone*

Time at reflux (min.)	Recovery of cortisone*	
	As Zn/17-KGS (%)	As 17-KGS† (%)
0	—	79
10	46	—
20	30	79
30	29	77
45	21	81
60	20	75
90	18	71

* Recoveries calculated on the basis of quantitative conversion into androst-4-ene-3:11:17-trione and that compound's colorimetric equivalence to 0·97 mole of dehydro*epi*androsterone (see Brooks & Norymberski, 1953).

† Controls without addition of zinc.

whichever applicable. In control experiments the addition of Zn was omitted. The products were submitted to one or more of the following analytical tests: (*a*) measurement of extinction at maximum absorption of starting material (237–242 mμ.); (*b*) measurement of Porter–Silber chromogens; (*c*) measurement of Zimmermann chromogens; (*d*) as (*c*) after treatment with bismuthate; and (*e*) as (*d*) after reduction with borohydride. All experiments except those under (*a*) were duplicated. The results (Table 4) are expressed in proportion to values found for corresponding control samples.

The reaction product obtained from cortisol, and to a lesser extent that obtained from its acetate, exhibited with the Porter–Silber reagent a strong absorption at 430 mμ. This observation cannot, however, indicate that the product is largely composed of a compound with an intact dihydroxyacetone grouping, since (i) its 17-KGS value was only 19% of that of cortisol (see Table 4), (ii) its absorption (with the Porter–Silber reagent) between 320 and 500 mμ. differed significantly from that of cortisol (see Fig. 3), and (iii) already with a tenfold excess of Zn cortisol was converted into corticosterone in high yield (Norymberski, 1956). It is therefore concluded that, under the conditions here employed, cortisol is transformed into a compound which, although a Porter–Silber chromogen, does not contain the dihydroxyacetone grouping.

Treatment of urine specimens with zinc. All specimens were from 24 hr. collections. Each was diluted with water to 2·0 l. and stored at 4°.

General procedure. A mixture of the urine (3·0 ml.), acetic acid (3·0 ml.), and Zn powder (0·6 g.; AnalaR) is kept at reflux for 1 hr. After cooling to room temperature a sample (4·0 ml.) of the supernatant is treated first with $NaBiO_3$ and then with HCl exactly as previously described for the determination of total 17-KS (Gibson & Norymberski, 1954). The total 17-KS and 17-KS are determined concurrently.

Variation of reaction time. Two urine specimens were treated by the general procedure except that the time of reaction with Zn was varied between 30 and 180 min. 17-KS and total 17-KS were determined in duplicate: the results are in Table 5. With one of the specimens control experiments were carried out in which the addition of Zn was omitted: it was found that boiling for up to 90 min. in the presence of acetic acid had no effect on the total 17-KS values of the urine.

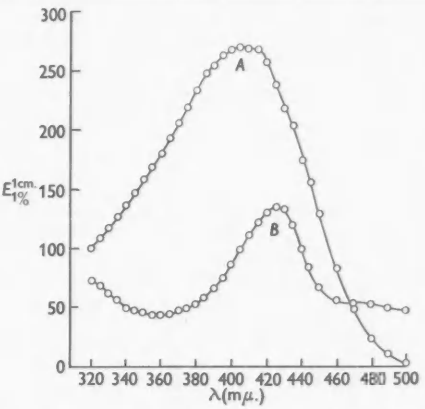


Fig. 3. Chromogenic (Porter–Silber) properties of cortisol and its transformation product with zinc. Curve *A*: cortisol. Curve *B*: reaction product.

Table 4. *Some properties of model compounds treated with zinc*

Compound treated	Ratio of extinction of product to that of starting material (%): At $\lambda_{max.}$ (237–242 mμ.)	At 430 mμ. with Porter–Silber reagent	At 520 mμ. (corrected) with Zimmermann reagent: Direct	After BiO_3^- treatment	After BH_4^-/BiO_3^- treatment
17α:21-Dihydroxypregn-4-ene-3:11:20-trione (cortisone)	4	2	—	25	—
Cortisone acetate	4	2·5	—	—	—
11β:17α:21-Trihydroxypregn-4-ene-3:20-dione (cortisol)	6	61	—	19	—
Cortisol acetate	0·3	19	—	—	—
21-Acetoxy-17α-hydroxypregn-4-ene-3:20-dione	2	2	—	—	—
21-Hydroxypregn-4-ene-3:20-dione	3	—	—	—	—
Pregn-4-ene-3:20-dione	0·6	—	—	—	—
11α-Hydroxypregn-4-ene-3:20-dione	2·5	—	—	—	—
Pregn-4-ene-3:11-20-trione	4	—	—	—	—
Androst-4-ene-3:17-dione	4	—	105	—	—
Androst-4-ene-3:11:17-trione	2	—	—	—	—
11β-Hydroxyandrost-4-ene-3:17-dione	0·3	—	—	—	—
3β-Hydroxyandrost-5-en-17-one	—	—	110	—	—
3α:17α:20β-Trihydroxypregnan-11-one	—	—	—	102	—
17α:20β:21-Trihydroxypregn-4-en-3-one	—	—	—	82	—
3α:17α-Dihydroxypregnane-11:20-dione	—	—	103	99	94
21-Acetoxy-3α:17α-dihydroxypregnane-11:20-dione	—	—	250	110	134

Addition of model compounds to the urine. Cortisone (10–50 μg./ml.), 3α:17α:20β-trihydroxypregnan-11-one (18·2 μg./ml.), and 17α:20β:21-trihydroxypregn-4-en-3-one (19·8 μg./ml.) were added to urine samples, which were then assayed for 17-KGS and Zn/17-KGS. Treatment with Zn reduced the conversion of cortisone into a Zimmermann chromogen from 78–88 to 7–10%, but failed to affect the 17-ketogenic property of the two other model compounds (see Table 6).

Thirty-four randomly chosen urine specimens were assayed for Zn/17-KGS. The results were in the range of 1·3–12·2 μg./ml. (mean 5·3 μg./ml.). After the addition of cortisone (10 μg./ml.) to each specimen the average increment of Zn/17-KGS was 0·45±0·85 μg./ml. This increment was found to be statistically significant at the 99% probability level.

Treatment of pre-reduced urines with Zn. Each of three urine specimens was reduced with borohydride, then treated with Zn by the general procedure and finally oxidized with bismuthate. In parallel experiments the treatment with Zn was omitted. Each determination was carried out in quadruplicate. In Table 7 the results are given as mean values and their standard deviations.

Precision of routine assays. 17-KS, total 17-KS, Zn/total 17-KS, and 17-hydroxycorticosteroids (17-OH CS; cf. Appleby *et al.* 1955) were determined in duplicate in eighty-two randomly chosen urine specimens. Statistical evaluation of the results (see Table 8) shows that treatment with Zn does not seem to introduce a new systematic error but that the percentage error of the derived values for Zn/17-KGS and SDHA is considerable.

Table 5. *Determination of* 17-*KS and total* 17-*KS in urine treated with zinc for various times*

Reaction time (min.)	17-KS (μg./ml.)		Total 17-KS (μg./ml.)	
	Urine A	Urine B	Urine A	Urine B
0	20·6	8·4	35·8	14·5
30	20·6	9·2	30·3	13·2
60	20·0	8·6	27·5	11·8
90	—	8·5	—	11·5
120	20·6	—	27·7	—
180	19·7	—	27·4	—

Table 6. *Contributions of model compounds to the determination of* 17-*KGS and* Zn/17-*KGS in the urine*

Steroid added to the urine (μg./ml.)	Recovery	
	As 17-KGS (%)	As Zn/17-KGS (%)
Cortisone: 10	79	10
20	88	7
30	80	10
40	79	10
50	78	7
3α:17α:20β-Trihydroxy-pregnan-11-one: 18·2	98	103
17α:20β:21-Trihydroxy-pregn-4-en-3-one: 19·8	92	99

Table 7. *Treatment of pre-reduced urines with zinc*

Urine	Zimmermann chromogens in terms of dehydro*epi*androsterone after treatment with	
	BH_4^-/BiO_3^- (mg./24 hr.)	$BH_4^-/Zn/BiO_3^-$ (mg./24 hr.)
C	15·1±0·4	15·2±1·0
D	41·8±2·6	40·1±2·2
E	16·7±1·3	16·9±0·6

DISCUSSION

The functional groups of urinary steroids comprise olefinic bonds, hydroxyl groups and keto groups. As far as could be ascertained (cf. Nes & Mason, 1951; Ercoli & Ruggieri, 1952; Fieser, 1953; Sondheimer, Kaufman, Romo, Martinez & Rosenkranz, 1953; Amandolla, Rosenkranz & Sondheimer, 1954; Barton & Robinson, 1954; Rosenfeld & Gallagher, 1955; Norymberski, 1956; this paper) any one of these functions in isolation resists the action of zinc in glacial or aqueous acetic acid even under the most vigorous conditions (prolonged heating at reflux and large excess of the reagent). However, when any two of these functions are placed at adjacent carbon atoms, the resulting groupings (α-ketols, allylic alcohols, α:β-unsaturated ketones) can be brought to react with zinc under appropriate conditions. Though an exception has been found in the behaviour of 17:20-ketols and other exceptions are possible, the generalization is useful in indicating which urinary steroids are

Table 8. *Statistical evaluation of eighty-two duplicate assays*

	Mean (μg./ml.)	Range (μg./ml.)	Standard deviation (s)* (μg./ml.)	Coeff. of variation (%)
Total 17-KS (a)	16·3	7·2–52·0	0·85	5
Zn/total 17-KS (b)	12·3	5·9–43·7	0·765	6
17-KS (c)	6·7	2·8–22·5	0·195	3
17-OH CS (d)	10·0	3·8–38·6	0·57	6
17-KGS (e)	9·6	3·0–42·0	0·86	9
Zn/17-KGS (f)	5·6	1·4–21·2	0·78	14
SDHA (g)	4·0	0 –28·0	1·13	28

* s_a, s_b, s_c and s_d were calculated as $\frac{\text{average within sample range}}{1{\cdot}13}$; $s_e=\sqrt{(s_a^2+s_c^2)}$; $s_f=\sqrt{(s_b^2+s_c^2)}$; $s_g=\sqrt{(s_a^2+s_d^2)}$.

likely to undergo transformations when the urine is treated with zinc, and how such transformations will affect the proposed differentiation of urinary 17-ketogenic steroids. For instance, if it is assumed that urinary α-ketols other than 17:20-ketols are converted by zinc into the corresponding ketones and by bismuthate into carboxylic acids, then consecutive treatment with zinc and bismuthate will give rise to an increase of ketonic components in the neutral fraction of the reaction products and hence to an increase of Zimmermann chromogens measured in the assay of Zn/17-KGS. The latter increase will be relatively large if the products obtained from α-ketols are 17-oxosteroids.

For reasons stated above, and because dihydroxyacetones on treatment with zinc and then with bismuthate retain some chromogenic property in the Zimmermann reaction, the assay of Zn/17-KGS will tend to overestimate the urinary levels of 17:20-glycols and 17:20:21-triols, and consequently the values calculated for urinary dihydroxyacetones will underestimate the actual amount of this group of compounds. Further, in its present form, the proposed assay suffers from a lack of precision (see Table 8). Nevertheless the results so far obtained with its application to clinical material (see Fig. 3) clearly demonstrate its usefulness.

SUMMARY

1. Zinc powder in boiling aqueous acetic acid was found to effect the reductive removal of (i) the keto group in 4:5-unsaturated 3-oxosteroids, (ii) the 17- and 21-hydroxyl groups in 17α:21-dihydroxy-20-oxosteroids, and (iii) the 21-acetoxyl group in 21-acetoxy-17α-hydroxy-20-oxosteroids. Among the types of compounds resistant to the action of zinc were 17-ketones, 17:20-diols and 17:20:21-triols.

2. An analytical method based on these findings was developed for the differential estimation of zinc-resistant (17:20-diols and 17:20:21-triols) and zinc-labile (dihydroxyacetones) 17-ketogenic steroids.

3. Urine specimens obtained from men and women in normal health, women in the last trimester of pregnancy, rheumatoid arthritics receiving cortisone acetate or corticotrophin and patients with adrenogenital syndrome were assayed by the analytical method described.

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Indirect Analysis of Corticosteroids

4. A NOTE ON THE ROLE OF CHLORIDE AND UREA IN THE BISMUTHATE TREATMENT OF URINES

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Smith & Tompsett (1955) recently described some reactions of sodium bismuthate in an aqueous acetic acid (50 %, v/v) solution of sodium chloride (0·75 %, w/v). They found that the reaction product obtained under these conditions from androstanediol (presumably 5α-androstane-3β:17β-diol) was strongly chromogenic in the Zimmermann reaction. Under identical conditions *allo*pregnanediol (3β:20α-diol?) gave a weakly chromogenic product. These results were demonstrated to depend upon the presence of chloride, and were interpreted as arising from the oxidation of chloride to chlorine and the latter agent's ability to oxidize 17-hydroxyandrostanes and 20-hydroxypregnanes to the corresponding ketones. In another group of experiments the authors submitted extracts of urinary steroid conjugates (Edwards, Kellie & Wade, 1953) to treatment with bismuthate followed by hydrolysis with hot hydrochloric acid. They found that the presence of chloride during the oxidative step caused a substantial decrease of the isolated chromogens, and they attributed this finding to an increased destructive effect during the hydrolytic procedure 'due to the formation of unstable chlorine addition or oxidation products'. They concluded that, since urine contains varying and often considerable (up to 1 %) amounts of sodium chloride, treatment of the urine with sodium bismuthate followed by acid hydrolysis will introduce the following two variable factors affecting the measurement (as Zimmermann chromogens) of native 17-oxosteroids and those formed from 17-hydroxycorticosteroids: (i) the formation of additional chromogens by oxidation of native 17-hydroxyandrostanes, and (ii) the destruction of chromogens during the hydrolytic procedure. The purpose of the present communication is to examine Smith & Tompsett's (1955) conclusions in the light of data already published and those now presented.

The reliability of the treatment of urine with sodium bismuthate as a means of converting native 17-hydroxycorticosteroids into 17-oxosteroids or of cleaving glucuronides was tested by criteria designed to reveal any gross interference with the specificity or efficacy of the reagent. The following instances may be cited.

(i) Addition to the urine of a 17-ketone (dehydro-*epi*androsterone), or of various 17-hydroxycorticosteroids followed by treatment with bismuthate (or by a sequence of reactions terminating with bismuthate treatment) and then by acid hydrolysis, in all cases resulted in the isolation of an amount of Zimmermann chromogens reasonably close to that expected and comparable to that obtained in experiments carried out in aqueous acetic acid (Norymberski, 1952; Norymberski, Stubbs & West, 1953; Norymberski & Stubbs, 1954; Appleby, Gibson, Norymberski & Stubbs, 1955; Norymberski & Stubbs, 1956). Clearly, the destructive effect of urinary chloride was in no case manifested.

(ii) When a pregnancy urine was treated with sodium bismuthate and then extracted, pure pregnane-3α:20α-diol was isolated in the same yield as from a sample of the same urine that was hydrolysed with hydrochloric acid (Norymberski & Sermin, 1953). Here then, any release of chlorine from urinary chloride failed to oxidize the 20-hydroxyl group.

(iii) Addition of dehydro*epi*androsterone to eleven randomly chosen urines, followed by treatment with borohydride, then with bismuthate, and finally with hot hydrochloric acid, in no case produced a significant increase in the chromogenic values of the extracts (Appleby *et al.* 1955). Oxidation by chlorine of androst-5-ene-3β:17β-diol (from reduc-

Table 1. *Determination of* 17-*oxosteroids in bismuthate-treated urine after addition of androst-5-ene-*3β:17β-*diol and* NaCl

Added to the urine		
Androst-5-ene-3β:17β-diol (μg./ml.)*	NaCl (%, w/v)*	Chromogens found (μg./ml.)†
0	0	21·8±2·6
100	0	22·0±1·0
100	0·5	22·8±1·0
100	1·0	20·5±1·3
100	2·5	21·1±0·8
100	5·0	21·1±0·8

* Concentration in the urine.

† Mean values of four measurements and standard deviations in terms of dehydro*epi*androsterone.

Table 2. *Determinations of* 17*-oxosteroids in bismuthate-treated urine after addition of dehydro*epi*androsterone and* NaCl

Added to the urine			
Dehydro*epi*androsterone (μg./ml.)*	NaCl (%, w/v)*	Chromogens found (μg./ml.)†	Recovery of dehydro*epi*androsterone (μg./ml.)
0	0	18·4±1·1	—
0	1·0	19·6±0·9	—
22·5	0	37·0±3·3	18·6±3·5
22·5	1·0	37·5±2·2	18·1±2·4

* Concentration in the urine.

† Mean values of six measurements in terms of dehydro*epi*androsterone.

Table 3. *Effect of urea on the oxidation of androst-5-ene-*3β:17β*-diol by bismuthate in the presence of chloride*

NaCl* (%, w/v)	Urea* (%, w/v)	Zimmermann chromogens† (μg./sample‡)
0	0	2·2±0·6 (4)§
0·3	0	27·0±0·5 (4)
0·6	0	36·3±0·5 (4)
0·9	0	42·5±1·2 (4)
1·2	0	48·6±1·9 (4)
1·5	0	47·2±4·2 (16)
1·5	0·05	45·0±1·3 (4)
1·5	0·10	29·9±1·8 (4)
1·5	0·15	7·6±0·9 (4)
1·5	0·20	3·9±0·5 (4)§
1·5	0·25	3·7±1·4 (8)§
1·5	0·50	4·4±0·4 (4)§
1·5	0·75	4·7±0·4 (4)§
1·5	1·0	4·0±0·8 (8)§
1·5	1·5	3·4±1·7 (8)§
1·5	2·0	3·9±1·2 (8)§
1·5	2·5	4·0±1·0 (8)§
1·5	3·0	3·8±1·6 (8)§

* Concentration in 50% acetic acid.

† Mean values and standard deviations in terms of dehydro*epi*androsterone; figures in parentheses denote numbers of replicate measurements.

‡ Corresponding to 80μg. of treated androst-5-ene-3β:17β-diol.

§ Atypical colour.

Table 4. *Bismuthate oxidation of cortisone in the presence of urea or* NaCl *or both*

NaCl* (%, w/v)	Urea* (%, w/v)	Recovery of cortisone† (%)
0	0	84±9
0	3	91±7
1·5	0	95±6
1·5	3	95±7

* Concentration in 50% acetic acid.

† Calculated on the basis of the compound's conversion into androst-4-ene-3:11:17-trione; mean values and standard deviations from six measurements of Zimmermann chromogens.

tion of added dehydro*epi*androsterone with borohydride) was not apparent.

Unless the unlikely assumption is made that all urines employed in these experiments contained subnormal amounts of chloride, the evidence cited shows that urinary chloride does not exert the types of interference predicted by Smith & Tompsett. More direct and conclusive evidence has now been obtained by adding androst-5-ene-3β:17β-diol and sodium chloride to a urine specimen subsequently treated with bismuthate and then with hot hydrochloric acid. The results (see Table 1) give no indication of the oxidation of the diol to a 17-ketone. Further, the recovery of dehydro*epi*androsterone from a urine specimen treated as above was unaffected by addition of chloride (see Table 2).

Urea, the major solute of the human urine, is known to undergo the Hofmann amide reaction with hypochlorites (Schestakow, 1905). It seemed to us that, when present in the system $NaBiO_3$–NaCl–$CH_3.CO_2H$–H_2O, urea may well compete for the effective non-specific oxidizing agent (probably hypochlorite) and consequently inhibit its other reactions, without affecting the specific glycol-cleaving action of bismuthate. This hypothesis was tested by treating androst-5-ene-3β:17β-diol with the reagent system given above in the presence of urea. The results (see Table 3) clearly demonstrate that relatively small quantities of urea (0·13 mole/mole of NaCl) effectively inhibit the oxidation of the diol to a 17-ketone. It is reasonable to assume that the native urea of the human urine plays the same protective role when the urine is treated with bismuthate. Finally it was shown that the presence of urea or chloride, or both, had no significant effect on the formation of Zimmermann chromogens from cortisone treated with bismuthate (see Table 4).

All experimental procedures were essentially as previously described (see preceding paper and references cited therein). They were performed on samples of urine (2·0 ml.) diluted with an equal volume of acetic acid, or on samples (2·0 ml.) of aqueous acetic acid (50%, v/v) solutions, whichever were applicable.

SUMMARY

1. The oxidation of 17-hydroxyandrostanes to 17-ketones in the system $NaBiO_3$–NaCl–$CH_3.CO_2H$–H_2O (Smith & Tompsett, 1955) was confirmed. It was shown that urea effectively inhibits this reaction.

2. Treatment of the human urine with sodium bismuthate in the presence of acetic acid, followed by the reductive removal of surplus reagent and then by treatment with hot hydrochloric acid, in no case resulted in the oxidation of 17-hydroxyandrostanes to 17-ketones or in the destruction of

17-ketones. The failure of urinary chloride to induce these reactions was attributed to the inhibitory effect of native urea.

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Factors which Interfere in the Manometric Assay of Monoamine Oxidase

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The reaction which monoamine oxidase is believed to catalyse may be represented by

$$R.CH_2.NH_2 + H_2O + O_2 \rightarrow R.CHO + H_2O_2 + NH_3,$$

in which one molecule of oxygen is absorbed for each molecule of substrate oxidized. When tyramine is used as substrate the theoretical oxygen uptake is apparently obtained, the observed value in the present experiments being 1·9 atoms/molecule of tyramine. Previous authors (Kohn, 1937; Pugh & Quastel, 1937; Blaschko, Richter & Schlossman, 1937; Luschinsky & Singher, 1945) have pointed out that crude monoamine oxidase preparations also contain catalase, cytochrome *c* and cytochrome oxidase, aldehyde oxidase and peroxidase, enzymes whose combined action (see Fig. 1) would be

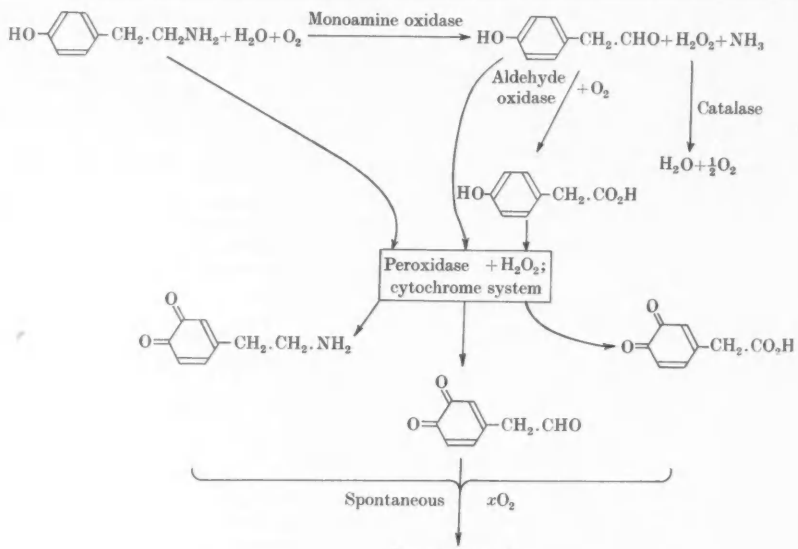

Fig. 1. Reactions likely to occur when tyramine is incubated with crude monoamine oxidase.

expected to make the observed oxygen uptake different from the theoretical value, and seriously to interfere with monoamine oxidase determinations based upon oxygen-uptake measurements.

It was shown by Blaschko *et al.* (1937) and Kohn (1937) that the side oxidations could be inhibited and the oxygen uptake with tyramine as substrate decreased to one atom/molecule by the addition of 10^{-2} M semicarbazide and 10^{-3} M cyanide. This value is only one-half of that to be expected if peroxidase, catalase, aldehyde oxidase and cytochrome oxidase were inhibited.

As the manometric method was needed for routine assays of the monoamine oxidase activity of mitochondrial preparations it was considered advisable to investigate the reasons for this discrepancy before placing any reliance on the results. Experiments were therefore performed to discover (*a*) whether the interfering enzymes indicated in Fig. 1 were in fact present in mitochondrial suspensions and (*b*) how their activity would be affected by various inhibitors.

EXPERIMENTAL

Mitochondrial suspensions. Mitochondria were separated from rat-liver homogenates by the method of Hawkins (1952), washed twice with distilled water and suspended in distilled water so that 2 ml. of suspension was equivalent to 1 g. of fresh liver. This preparation was free from blood pigments and soluble protein.

Oxygen uptake. Uptake of O_2 during deamination was measured manometrically at 38°, readings being taken at 0, 10, 20 and 30 min. for an activity determination and, when total O_2 uptake was being measured, at 30 min. intervals thereafter until O_2 ceased to be absorbed. The composition of the basic reaction mixture was as follows. Main compartment: enzyme preparation (1 ml.), 0·24 M sodium phosphate buffer (pH 7·0; 0·2 ml.), water as necessary; side arm: 0·1 M amine (0·2 ml.); centre well: 2 M-KOH (0·1 ml.), filter-paper strip; if KCN was present in the main compartment 2 M-KOH was replaced by 2 M-KCN, which was sufficiently alkaline to absorb CO_2. The final volume of the reaction mixture was adjusted to 2 ml. with water. Additions to this mixture were made by replacing some of the water in the main compartment, all solutions except 2 M-KOH and 2 M-KCN being adjusted to pH 7·0 before use. The flasks were gassed with O_2 for 2 min. and equilibrated for 10 min. before tipping. A control reaction mixture containing no substrate was also prepared and any O_2 absorbed by it was subtracted from that absorbed in the test reaction mixtures.

Catalase. This was measured by the following modification of the method of Goldacre & Galston (1953). The reaction mixture contained: 0·24 M sodium phosphate buffer, pH 7·0 (1 ml.), mitochondrial suspension (2·5 ml.), 0·1 M-H_2O_2 (1 ml.) and water to 10 ml. The reaction was carried out in boiling tubes incubated at 38° and was started by adding the H_2O_2. Samples (2 ml.) were removed at 15, 30, 45 and 60 min. and transferred to tubes containing 2 ml. of 5% trichloroacetic acid + 0·5 ml. of 12 N-H_2SO_4. Precipitated protein was removed by filtration, the precipitate washed with water and the H_2O_2 in the combined filtrate and washings titrated with 0·1 M-$KMnO_4$. The residual H_2O_2 was expressed as a percentage of that originally present and plotted against the time of sampling. The effect of cyanide on catalase activity was studied by replacing some of the water in the reaction mixture by cyanide solution adjusted to pH 7·0.

Peroxidatic activity of catalase. This was demonstrated by adding 0·2 ml. of 4% ethanol to the main compartment of the Warburg flask in the basic reaction mixture.

Cytochrome oxidase. This was measured by the method of Schneider & Potter (1943), in which O_2 uptake is followed during the oxidation of ascorbic acid by a mixture of cytochrome oxidase and cytochrome *c*. The effects of inhibitors were investigated by including appropriate solutions in the main compartment of the Warburg flask.

Aldehyde oxidase. This was measured by the method of Walkenstein & Weinhouse (1953), in which O_2 uptake is followed when mitochondria are incubated at 37° and pH 7·4 with 0·01 M aldehyde, 0·1 M-KCl, 0·005 M-$MgSO_4$, 10^{-4} M fumarate, 10^{-3} M adenosine triphosphate (ATP) and 0·02 M sodium phosphate buffer.

Chemicals. These were British Drug Houses, Ltd. laboratory grade except Antabuse (tetraethylthiuram disulphide) from L. Light and Co. Ltd., ATP (Boots Ltd.), and Marsilid (1-*iso*nicotinyl-2-*iso*propylhydrazine) from Roche Products Ltd.

RESULTS

Cytochrome oxidase

An active cytochrome oxidase was found to be present in the washed mitochondrial suspension. The curves in Fig. 2 show that in the conditions used the activity was dependent on the concentration of added cytochrome *c*, in the absence of which cytochrome oxidase activity could not be observed. This agrees with the results of Friedenwald & Herrmann (1942) who also found that tissue preparations could readily be obtained which contained abundant cytochrome oxidase but relatively little cytochrome *c*.

Cyanide (10^{-3} M) was found to inhibit the enzyme completely (Fig. 2); however, it it unlikely that this would explain the effect of cyanide in lowering the O_2 uptake in the basic reaction mixture since the cytochrome oxidase of washed mitochondria is almost inactive in the absence of added cytochrome *c*.

Semicarbazide (0·01 M) did not affect mitochondrial cytochrome oxidase and its ability to lower the O_2 uptake during the deamination of tyramine must therefore be due to some other effect (Fig. 2).

When 10^{-4} M Marsilid, a potent monoamine oxidase inhibitor (Zeller & Barsky, 1952) which does not inhibit cytochrome oxidase, was added to the basic reaction mixture the O_2 uptake was reduced to zero. This indicates that, even in the absence of cyanide, oxidation of tyramine by the cytochrome system (see Fig. 1) does not occur.

The evidence is therefore against the cytochrome system being operative in washed mitochondria.

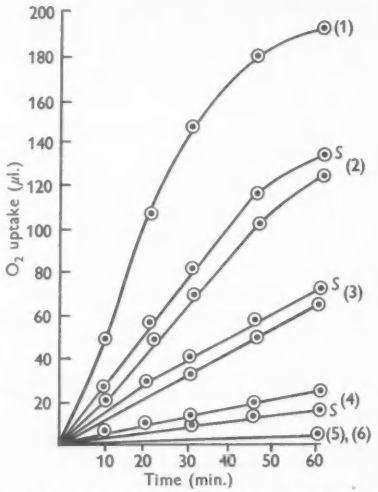

Fig. 2. Effect of 0·01 M semicarbazide on the cytochrome oxidase activity of washed mitochondria at different concentrations of cytochrome *c*. Mitochondrial suspension (1·0 ml.) was incubated at 37° with 0·2 ml. of 0·24 M sodium phosphate, pH 7·4, 0·2 ml. of 0·004 M-$AlCl_3$, 0·2 ml. of 0·114 M ascorbic acid, 0·1 ml. of 2 M-KOH on filter-paper strip, and the following additions: (1) cytochrome *c*, 1 mg./ml.; (2) cytochrome *c*, 0·3 mg./ml.; (3) cytochrome *c*, 0·1 mg./ml.; (4) cytochrome *c*, 0·01 mg./ml.; (5) cytochrome *c*, 1 mg./ml. $+10^{-3}$ M-KCN; (6) no cytochrome *c*. Curves marked '*S*' show the effect of adding 0·01 M semicarbazide. Final vol., 2 ml. Gas phase, O_2.

Aldehyde oxidase

The presence of aldehyde oxidase in washed mitochondria was suggested by the fact that the addition of semicarbazide to the basic reaction mixture, with tyramine as substrate, was accompanied by a marked fall in O_2 uptake. Blaschko *et al.* (1937) suggested that this was due to binding of the aldehyde formed during deamination and its consequent protection against attack by aldehyde oxidase.

Soluble aldehyde oxidase has been described by Gordon, Green & Subrahmanyan (1940), and by Racker (1949), and a diphosphopyridine nucleotide-dependent aldehyde oxidase associated with mitochondria has been described by Walkenstein & Weinhouse (1953). The soluble aldehyde oxidase would not be expected to remain associated with mitochondria washed with distilled water, and in view of the disruptive effects of distilled water on mitochondria it is possible that Walkenstein & Weinhouse's insoluble enzyme might be inactivated by removal of the diphosphopyridine nucleotide upon which its activity depends. All attempts to demonstrate aldehyde oxidase in washed mitochondria were without success, as the following experiments will show.

The absence of aldehyde oxidase in washed mitochondria was suggested by the fact that Antabuse (4 µg./ml.), which inhibits liver aldehyde oxidase (Kjeldgaard, 1949), was found to be without effect on the O_2 uptake when included in the basic reaction mixture.

Aldehyde oxidase activity in the mitochondrial suspension was then measured by the method of Walkenstein & Weinhouse (1953). Redistilled acetaldehyde, crotonaldehyde or *iso*valeraldehyde was used as substrate, and in no case was O_2 found to be absorbed over a period of 3 hr.

The failure to demonstrate the presence of aldehyde oxidase may have been due to (*a*) the presence of inhibitors in the aldehydes (Walkenstein & Weinhouse, 1953), or (*b*) the inactivation of the enzyme by a high initial concentration of aldehyde (Kjeldgaard, 1949). In the following experiment both of these factors were eliminated by supplying acetaldehyde continuously at low concentration by coupling the oxidation of ethanol with the production of H_2O_2 in the monoamine–oxidase reaction (see below).

Four reaction mixtures were set up, two without and two with semicarbazide. Ethanol was added to one of each pair and the O_2 uptake followed in each reaction mixture.

In the absence of semicarbazide the extra O_2 uptake resulting from the addition of ethanol includes O_2 used in the oxidation of ethanol to acetaldehyde by peroxidase, plus any that may be used in the subsequent oxidation of the acetaldehyde to acetic acid by aldehyde oxidase.

Table 1. *Effect of semicarbazide on the extra oxygen uptake resulting from coupling the oxidation of ethanol with the deamination of tyramine by monoamine oxidase*

Each vessel contained: side arm—0·1 ml. of 0·1 M tyramine; centre well—0·1 ml. of 2 M-KOH and a filter-paper slip; main compartment—1 ml. of mitochondrial suspension, 0·2 ml. of 0·24 M sodium phosphate buffer (pH 7·0) and, where present, 0·2 ml. of 4% (v/v) ethanol and 0·2 ml. of M or 0·1 M semicarbazide; total vol., 2 ml.; O_2 gas phase, temp. 38°.

Expt.	Semicarbazide (M)	Time (min.)	Extra O_2 uptake due to addition of ethanol (µl.) Semicarbazide absent	Semicarbazide present
1	0·01	100	87	106
		200	100	111
		300	113	120
2	0·10	100	86	94
		200	94	93

In the presence of semicarbazide, which traps the acetaldehyde and prevents its conversion into acetic acid, the extra O_2 absorbed after the addition of ethanol is used only in the oxidation of ethanol to acetaldehyde. Therefore, if aldehyde oxidase is present the extra O_2 uptake in the absence of semicarbazide should be greater than that in the presence of semicarbazide by a factor of approximately two. Alternatively, if aldehyde oxidase is absent, the extra O_2 absorbed after the addition of ethanol should be independent of the presence of semicarbazide. The experiment was performed with 0·1 M and 0·01 M semicarbazide; within the limits of error neither concentration was found to influence the extra O_2 absorbed after the addition of ethanol (Table 1). It was therefore concluded that aldehyde oxidase was absent.

Catalase and peroxidase

By the method of Goldacre & Galston (1953) a high concentration of catalase was found to be present in the washed mitochondria. Even in the presence of 0·05 M cyanide the catalase activity was not inhibited completely (Fig. 3). Semicarbazide (0·01 M) was found to be without effect on the catalase activity. Goldacre & Galston (1953) showed that 2×10^{-6} M 2:4-dichlorophenol would inhibit crystalline catalase by 50%; however, the results shown in Fig. 3 indicate that mitochondrial catalase activity is not affected by this inhibitor.

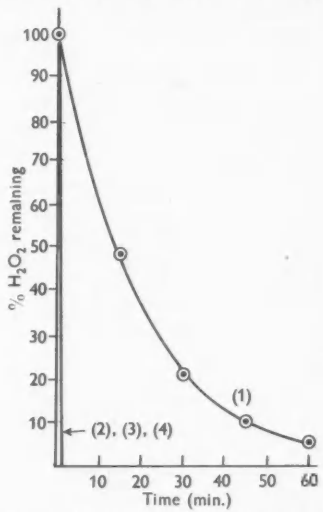

Fig. 3. Effect of cyanide or 2:4-dichlorophenol on the catalase activity of washed mitochondria. 0·01 M-H_2O_2 was incubated at 37° and pH 7·0 with mitochondrial suspension and (1) 0·05 M-KCN; (2) 10^{-3} M-KCN; (3) $0{\cdot}5 \times 10^{-4}$ M 2:4-dichlorophenol; (4) no inhibitor.

Table 2. *Effect of cyanide, azide or semicarbazide on the coupled oxidation of ethanol*

Each Warburg vessel contained: side arm—0·1 ml. of 0·1 M tyramine; centre well—0·1 ml. of 2 M-KCN or 2 M-KOH, filter-paper slip; main compartment—0·1 ml. of 0·24 M sodium phosphate buffer, pH 7·0, and the additions shown; total vol., 2 ml. O_2 gas phase, temp. 38°.

Additions (final concn.)	O_2 uptake* after 6 hr. (μl.)
None	210
Ethanol (0·4%, v/v)	324
Ethanol (0·4%, v/v) + KCN (0·001 M)	116
KCN (0·001 M)	116
Ethanol (0·4%, v/v) + NaN_3 (0·01 M)	210
NaN_3 (0·01 M)	210
Ethanol (0·4%, v/v) + semicarbazide (0·01 M)	217
Semicarbazide (0·01 M)	122

* Calc. for 1 atom of O_2/mole of tyramine = 112 μl.

The mitochondrial suspensions were found to be capable of coupling the formation of H_2O_2 during the deamination of tyramine with the oxidation of ethanol. This is in agreement with the experiments of Keilin & Hartree (1945), who showed that enzymically liberated H_2O_2 could be used in coupled oxidations.

This peroxidatic effect was demonstrated by adding 0·4% ethanol to the basic reaction mixture, when an increase in the O_2 uptake equivalent to one atom/molecule of substrate occurred. Similar experiments were performed by Kohn (1937), who used a crude monoamine oxidase preparation from pig liver. This increase in O_2 uptake on adding ethanol indicates that hardly any of the H_2O_2 liberated in the basic reaction mixture is used in coupled oxidations involving tyramine or its primary oxidation product. It must therefore be concluded that because of the absence of a suitable substrate the peroxidase effect does not operate during deamination of tyramine by mitochondria. The coupled oxidation was inhibited by 10^{-3} M cyanide or by 0·01 M azide but not by 0·01 M semicarbazide (Table 2).

Effect of various experimental conditions on O_2 uptake during monoamine oxidase activity

The effects of ethanol, azide, cyanide and semicarbazide on the total O_2 uptake during the deamination of tyramine or *iso*pentylamine were investigated. The results, together with the reactions which are thought to occur under each set of conditions, are illustrated in Table 3.

In some cases the observed O_2 uptake was greater than the theoretical based on the activity of enzymes shown to be present in the washed mitochondria. The extra O_2 is represented by x and was found to be equivalent to 0·8–0·9 atom/molecule of substrate.

Table 3. *Total O_2 uptake during the deamination of tyramine and* iso*pentylamine*

	Atoms of O_2/molecule of substrate					
	Tyramine (10^{-2}M)			*iso*Pentylamine (10^{-2}M)		
Combination*	Theoretical†	Observed	Reaction† involved	Theoretical	Observed	Reaction† involved
M	$1+x$	1·9	D.Ca.Sp	1	0·7	D.Ca
M.C	1	1·1	D.Ca	1	0·9	D.Ca
M.Sm	1	1·1	D.Ca	1	0·95	D.Ca
M.Sm.C	1	1·0	D.Ca	1	0·9	D.Ca
M.A	$1+x$	1·9	D.Ca.Sp	1	0·9	D.Ca
M.Et	$2+x$	2·9	D.P.Sp	2	2·3	D.P‡
M.Et.C	1	1·1	D.Ca	1	1·1	D.Ca‡
M.Et.Sm.C	1	1·0	D.Ca	1	1·1	D.Ca‡
M.Et.A	$1+x$	1·8	D.Sp	1	—	—
M.Et.Sm	2	1·9	D.P	2	2·1	D.P‡

* M, mitochondria; C, 10^{-3}M-KCN; Sm, 10^{-2}M semicarbazide; A, 10^{-2}M sodium azide; Et, 0·4% (v/v) ethanol.
† D, deamination; Ca, catalase; P, peroxidase; Sp, spontaneous oxidation; x, O_2 uptake due to spontaneous oxidation of *p*-hydroxyphenylacetaldehyde.
‡ In these cases O_2 uptake was not complete after 5 hr. and the O_2 absorbed at that time is therefore expressed as a fraction of the O_2 absorbed in a reaction mixture containing only mitochondria and buffered *iso*pentylamine (M).

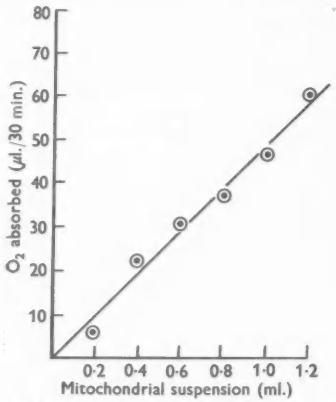

Fig. 4. Relation between enzyme concentration and O_2 uptake in the recommended monoamine oxidase activity test.

Method for routine monoamine oxidase determination

On the basis of the preceding experiments, monoamine oxidase activity was measured by following O_2 uptake during the deamination of tyramine in the presence of 10^{-2}M semicarbazide and 10^{-3}M cyanide. Under these conditions only monoamine oxidase and catalase appear to be active, spontaneous oxidations are inhibited, and O_2 is absorbed at the rate of one atom/molecule of tyramine deaminated. Tyramine is rapidly oxidized in comparison with other readily obtainable amines, O_2 uptake is linear over the first 30 min. of the reaction and is also directly proportional to the monoamine oxidase concentration (Fig. 4). The composition of the recommended reaction mixture was as follows: main compartment—enzyme preparation (1 ml.), 0·24M sodium phosphate buffer, pH 7·0 (0·2 ml.), 0·01M-KCN (0·2 ml.), 0·1M semicarbazide (0·2 ml.), water (0·2 ml.); side arm—0·1M tyramine (0·2 ml.); centre well—2M-KCN (0·1 ml.), filter-paper strip. All solutions except the 2M-KCN were adjusted to pH 7·0.

DISCUSSION

Of the enzymes included in Fig. 1 as being able to modify the uptake of oxygen during the deamination of tyramine by monoamine oxidase, cytochrome oxidase, peroxidase and catalase have been shown to be present in washed rat-liver mitochondria. Cytochrome oxidase was inactive because of the absence of sufficient cytochrome *c*, and peroxidase appeared to be inactive in the monoamine oxidase activity tests because of the absence of a suitable substrate. Aldehyde oxidase could not be detected in the washed mitochondria. Catalase was particularly active in all the mitochondrial preparations, and in a reaction mixture containing only buffered tyramine and washed mitochondria would be expected to reduce the oxygen uptake to one atom/molecule of substrate oxidized:

$$R.CH_2.NH_2 + H_2O + O_2 \xrightarrow{\text{monoamine oxidase}} R.CHO + H_2O_2 + NH_3$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2.$$

This was found not to be the case, however, the observed oxygen uptake being 1·9 atoms/molecule of substrate oxidized.

The addition of 10^{-3}M cyanide or 10^{-2}M semicarbazide to the basic reaction mixture decreased

the oxygen uptake to the theoretical value of one atom/molecule of tyramine oxidized. At the concentrations used these two substances are known not to inhibit either monoamine oxidase (Blaschko *et al.* 1937) or catalase. Other enzymes likely to be affected by 10^{-3} M cyanide were found to be absent from washed mitochondria or inactive under the conditions used for deamination. Semicarbazide (10^{-2} M) was found to be without effect on any of the enzymes against which it was tested. It would, therefore, appear that these substances may inhibit a spontaneous oxidation, possibly the spontaneous oxidation of *p*-hydroxyphenylacetaldehyde, the primary deamination product of tyramine. This compound is known to be unstable in air (Langheld, 1909) and both cyanide and semicarbazide could prevent its spontaneous oxidation; semicarbazide by the formation of a semicarbazone, and cyanide by heavy-metal binding or by cyanohydrin formation. The oxidation of this compound would account for the extra oxygen uptake observed in Table 3.

*iso*Valeraldehyde, the primary deamination product of *iso*pentylamine, was found not to be susceptible to further oxidation under the conditions of the monoamine oxidase assay; consequently, cyanide or semicarbazide would not be expected to lower the oxygen uptake during *iso*pentylamine deamination, and this was found to be the case (see Table 3). Attempts to prepare *p*-hydroxyphenylacetaldehyde were unsuccessful and its properties and behaviour under the conditions of the monoamine oxidase assay could, therefore, not be investigated.

SUMMARY

1. The occurrence in washed rat-liver mitochondria of enzymes likely to modify the oxygen uptake during the oxidative deamination of *iso*pentylamine or tyramine has been studied.

2. Catalase and peroxidase have been shown to be present and active. Peroxidase was inhibited by 0·01 M azide and by 10^{-3} M cyanide. Catalase was not inhibited by 2×10^{-6} M 2:4-dichlorophenol or by 0·01 M semicarbazide; it was incompletely inhibited by 0·05 M cyanide.

3. Cytochrome oxidase was present but inactive owing to the absence of sufficient cytochrome *c*. The enzyme was inhibited by 10^{-3} M cyanide.

4. Aldehyde oxidase could not be detected.

5. The effects of cyanide, semicarbazide, azide and ethanol on oxygen uptake during the deamination of tyramine and *iso*pentylamine have been studied, and the results discussed.

6. It is concluded that a buffered reaction mixture containing the monoamine oxidase preparation, 0·01 M tyramine, 0·01 M semicarbazide and 10^{-3} M cyanide would be suitable for measuring monoamine oxidase activity. Under these conditions one atom of oxygen was absorbed for each molecule of substrate oxidized and the oxygen absorbed at 30 min. was proportional to the monoamine oxidase concentration.

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A Study of the Effects of Substrate Concentration and Certain Relaxing Factors on the Magnesium-Activated Myofibrillar Adenosine Triphosphatase

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Marsh (1952) first showed that whereas the apparent rate of hydrolysis of adenosine triphosphate (ATP) by homogenates of fresh rabbit muscle in an isotonic solution of potassium chloride was comparatively low, after centrifuging and removal of the soluble protein extract a considerable increase in the rate of production of inorganic phosphate by the residue occurred when it was resuspended in fresh potassium chloride solution. On the basis of these findings Marsh concluded that there was present in muscle a factor which inhibited adenosine triphosphatase (ATPase) activity, and he further demonstrated that in the presence of this factor ATP caused an increase in the volume of the cell fragments in the suspension. Bendall (1953*a*) extended Marsh's findings and was able to bring about the relaxation of glycerated fibres by the application of ATP in the presence of preparations of the Marsh factor. He also confirmed the inhibitory action of the factor on the ATPase activity of homogenized glycerated fibres. Further investigations by Hasselbach & Weber (1953) on the ATPase activity of these preparations showed that at higher concentrations ATP was inhibitory, and in the presence of the Marsh factor the level at which ATP became inhibitory was considerably reduced.

It is clear from the investigations mentioned above and those of Bozler & Prince (1953) that, in general, relaxation in muscle models is associated with a low rate of ATP hydrolysis. Magnesium appears to be of special importance in the contraction–relaxation cycle, yet the precise role of this metal is far from clear. Although magnesium is essential for relaxation (Bendall, 1953*a*), reagents which form complexes with magnesium, namely ethylenediaminetetraacetate (EDTA) (Bozler, 1954; Watanabe, 1955) and inorganic pyrophosphate (Weber, 1951; Bozler, 1951; Bendall, 1953*b*) also bring about relaxation of glycerated fibres. Bendall (1953*b*) has commented on the similarity between the relaxing action of the Marsh factor and that of inorganic pyrophosphate, and it is conceivable that one of the properties possessed in common by these substances is the ability to complex with magnesium.

Glycerated fibres consist mainly of myofibrils, but studies on the ATPase activity of these fibres are not entirely satisfactory, as preparations may be contaminated with sarcoplasmic granules and nuclei which also possess ATPase activity, and with varying amounts of sarcoplasm. Furthermore, precise analysis of such experiments in terms of substrate and activator concentrations at any point in the fibre is difficult because concentration gradients will be produced by the diffusion of ATP to the myofibrils from the outside of the fibre. To throw more light on the enzymic behaviour of the myofibril a study has been made of the ATPase activity of isolated myofibrils, with particular reference to those conditions that bring about the low level of activity which is characteristic of relaxation. It has been shown that at a low ionic strength the marked inhibition of ATPase activity by ATP is dependent on the magnesium concentration and that this inhibition is not obtained when calcium is the activating metal, nor in the presence of magnesium when inosine triphosphate (ITP) is the substrate. No inhibition could be demonstrated with myokinase, but all the other relaxing factors tested reduced the ATPase activity of isolated myofibrils to a low level. With the exception of findings with EDTA the results are in agreement with the view (Perry, 1955, 1956) that the low rate of ATPase activity associated with relaxation may be due to the binding or removal of magnesium from the system, so that it is no longer available for the myofibrillar ATPase system.

A preliminary account of some aspects of this work has already been presented (Perry, 1955).

METHODS

Preparation of myofibrils

From fresh muscle. The method employed for large-scale preparations was similar to that reported earlier (Perry, 1952), except that the medium used (borate–KCl) contained 0·025 M-KCl and 0·039 M borate, pH 7·1. In this buffer less myofibrillar protein is leached out on standing, and the myofibrils are not so hydrated as those prepared in borate alone. For this latter reason the centrifuging times used differ from those originally given. All manipulations were

carried out at 0°, and preparations were controlled by frequent microscopical examination of the suspensions.

Chilled, minced, freshly excised rabbit muscle (200 g.) was homogenized for 1 min. with 5 vol. of borate–KCl and centrifuged for 15 min. at 600 *g*. The supernatant was discarded, the residue resuspended with a further 5 vol. of borate–KCl and homogenized for 2 min. After centrifuging for 15 min. at 600 *g* the supernatant was again discarded and the light-coloured upper layer of the sediment, consisting mainly of myofibrils, was removed with the aid of a little borate–KCl. The myofibril paste was diluted with borate–KCl to 1 l./200 g. of muscle taken, and coarse material removed by centrifuging for 3 min. at 400 *g*. The sediment was discarded and the suspension centrifuged again for 15 min. at 600 *g* to sediment the myofibrils. After three further resuspensions and centrifugings to remove sarcoplasmic proteins and granules the sediment was resuspended and centrifuged once more for 3 min. at 400 *g*. The sediment was again discarded and the turbid supernatant centrifuged to obtain a concentrated myofibril suspension. At this stage usually less than 1% of the protein was soluble. The myofibrils were transferred to a flask with a minimum amount of borate–KCl and stored at 0° in the presence of traces of toluene.

It was necessary, for some experiments, to prepare myofibrils rapidly from smaller amounts of muscle, e.g. 5 g. of rabbit psoas. Such preparations were carried out in borate–KCl and also in 0·1 M-KCl, although, in the latter medium, the endogenous ATP of the muscle appears to be more effective in causing contraction during homogenization. To minimize this effect the muscle was cut up with scissors and broken down into large cell fragments by homogenization with 20 vol. of medium for a few seconds in a small homogenizer of the Waring Blendor type fitted with an anti-foaming device. The suspension was centrifuged and the supernatant discarded. The residue was re-homogenized in 20 vol. of the medium until the cell fragments were almost completely broken down into myofibrils. After centrifuging the myofibrils were resuspended in 5–10 vol. of medium and the procedure was then similar to that followed in the large-scale preparation after the second homogenization. In solutions of ionic strength higher than that of the borate–KCl, myofibrils sedimented readily, and consequently lower speeds and shorter centrifuging times were used to remove unbroken cells and large myofibrillar bundles. It was advisable to follow this stage carefully by microscopical examination.

The stock suspensions contained 20–30 mg. of protein/ml. and were diluted with 4–5 vol. of borate–KCl for enzyme experiments. These preparations contained slight myokinase activity, which could be partly reduced by repeated washing and centrifuging. The myofibrils also possessed 5-adenylic-deaminase activity ($Q_{NH_3} = 200$–400 at pH 6·1 in succinate buffer at 20°) and very slight deaminating activity towards ADP. ($Q_{NH_3} = \mu$l. of NH_3/hr./mg. of protein.)

From glycerated fibres. Strips of rabbit psoas muscle which had been stored at −10° in 50% (v/v) aqueous glycerol were cut up into short lengths and homogenized with 20 vol. of the appropriate medium. The procedure was then as described above for the preparation of myofibrils from small amounts of rabbit muscle.

Myokinase

Myokinase was prepared by the method of Kalckar (1947) and stored at 0° as an $(NH_4)_2SO_4$ paste.

ATPase determinations

Incubations were usually carried out at 20·5° in a total vol. of 2 ml. containing 0·05 M aminotrishydroxymethylmethane (tris)–HCl buffer, pH 7·4, $CaCl_2$ or $MgCl_2$ as activator, ATP (Na salt), and other components as indicated. The reaction was started by the addition of 0·2 ml. of the myofibril suspension, and stopped by adding rapidly 1 ml. of 15% (w/v) trichloroacetic acid. The myofibril precipitate was removed by filtration through a loose pad of absorbent cotton wool, and phosphate estimated by the method of Fiske & Subbarow (1925) in a final vol. of 25 ml.

Low concentrations of inorganic pyrophosphate inhibit the development of colour under the conditions of the Fiske & Subbarow (1925) method, and in all experiments reported here care was taken to ensure that the final inorganic pyrophosphate concentration in the solution used for colorimetric measurements did not exceed 1 mM. At this concentration no inhibition of colour development was obtained, although 2 mM-$Na_4P_2O_7$ was found to give marked inhibition. Magnesium chloride did not have any significant effect on the final development in the presence of $Na_4P_2O_7$ or ATP in the concentrations used in the enzyme experiments.

Nucleotides

ATP, adenosine diphosphate (ADP), ITP and uridine triphosphate (UTP) were the Na salts (Sigma Chemical Co.). Neutralized solutions (0·05 M) were stored at −10°. Concentrations of the triphosphates were determined by assuming that 7 min. hydrolysis in N-HCl at 100° liberated two-thirds of the total organic P as inorganic P. For ADP the acid-labile P was taken as equal to half the total organic P. Chromatography with the *iso*butyric acid–ammonia solvent of Krebs & Hems (1953) indicated that the ATP was pure, whereas the ITP contained a trace of inosine diphosphate, and the UTP a small amount of another nucleotide which was probably uridine diphosphate.

Inosine monophosphate (IMP) was prepared from 5-adenylic acid (L. Light and Co. Ltd.) by the method of Embden & Schmidt (1929).

RESULTS

Effect of ATP concentration on the ATPase activity of isolated myofibrils

When $MgCl_2$ was the activator and in the absence of salts other than those present in the standard incubation medium (ionic strength, I, approx. 0·06) higher ATP concentrations caused a marked inhibition of the myofibrillar ATPase. The substrate level at which the ATPase activity began to decline was dependent on the $MgCl_2$ concentration (Fig. 1). In general, the results suggested that at low ionic strength inhibition began when the concentration of ATP exceeded that of $MgCl_2$. This was clearly so over the range 5–10 mM-$MgCl_2$, but with 2–3 mM-$MgCl_2$ the lowest relative concentrations of ATP required to produce inhibition were somewhat higher.

It is possible that this divergence at the lower range of $MgCl_2$ concentrations was due to small amounts of magnesium which may be present in the

myofibril preparations. The fact that well-washed isolated myofibrils possess appreciable ATPase activity in the absence of added $MgCl_2$ suggests that activating cations are still present in these preparations.

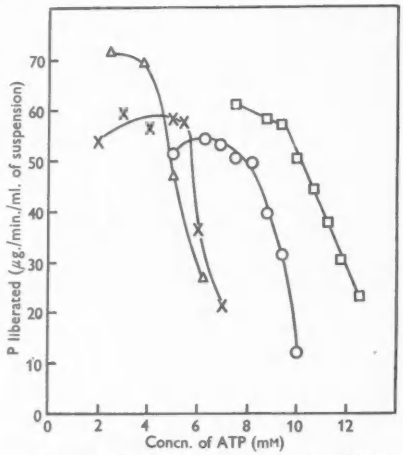

Fig. 1. Effect of $MgCl_2$ concentration on the inhibition of the myofibrillar ATPase activity by excess of substrate. Incubations were carried out in 0·05 M tris buffer (pH 7·4) for 5 min. at 20·5°, and a different myofibril preparation was used for each level of $MgCl_2$: △, 2·5 mM-$MgCl_2$; ×, 5 mM-$MgCl_2$; ○, 7·5 mM-$MgCl_2$; □, 10 mM-$MgCl_2$.

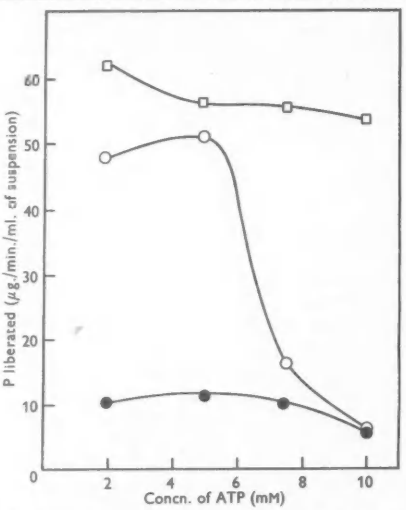

Fig. 2. Comparison of activation of myofibrillar ATPase by $MgCl_2$ and $CaCl_2$ with increasing ATP concentration. Experiment carried out as those illustrated in Fig. 1. ●, No activator; ○, 5 mM-$MgCl_2$; □, 5 mM-$CaCl_2$.

With 5 mM-$MgCl_2$ similar substrate-inhibition effects were obtained in 0·05 M glyoxaline buffer pH 6·9 and 0·05 M tris buffer pH 7·7.

The inhibitory action of higher concentrations of ATP was shown to be reversible in that addition of $MgCl_2$ to systems containing excess of substrate restored the enzymic activity to a high level. At pH 7·4, with 5 mM ATP and 2 mM-$MgCl_2$, or with 7·5 mM ATP and 3·75 mM-$MgCl_2$, the activity was low. On addition of $MgCl_2$ to bring the concentration equal to that of the substrate in each case the level of ATPase activity rose to approach that obtained in control experiments in which the ATP and $MgCl_2$ concentrations were equal throughout.

Inhibition by higher concentrations of substrate appeared to be characteristic of magnesium activation of the myofibrillar ATPase, for when $MgCl_2$ was replaced by 5 mM-$CaCl_2$ a very slight decrease in activity was obtained on increasing the ATP concentration over the range 2–10 mM (Fig. 2). Furthermore, under conditions in which the magnesium-activated ATPase was inhibited by excess of substrate, addition of Ca abolished the inhibition. In fact, with certain concentrations of $CaCl_2$ the ATPase activity may rise with increasing ATP concentration, as is shown in Fig. 3, which illustrates the results of experiments carried out in the presence of 5 mM-$MgCl_2$ and $CaCl_2$ ranging from 0·5 to 5 mM. It seems clear that this effect is not

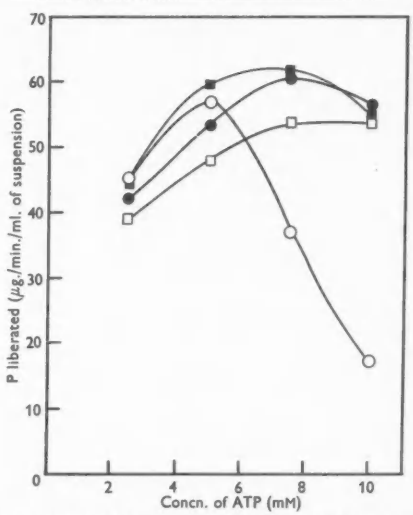

Fig. 3. Effect of $CaCl_2$ on the substrate inhibition of Mg-activated myofibrillar ATPase. Conditions of enzyme experiment similar to those of Fig. 1, with 5×10^{-3} M-$MgCl_2$ present in all cases and the following further additions: ○, none; ■, 0·5 mM-$CaCl_2$; ●, 2 mM-$CaCl_2$; □, 5 mM-$CaCl_2$.

simply due to the activating effect of $CaCl_2$ alone, for whereas 0·2 mM-$CaCl_2$, in the presence of $MgCl_2$, restores the activity to the level obtained when ATP is not in excess, this concentration of $CaCl_2$ produces little activation of the myofibrillar ATPase when it is the only added activator (Fig. 4).

Effect of ionic strength. On increasing the ionic strength of the incubation medium to approx. 0·16 by the addition of KCl, the activity of the magnesium-activated ATPase of myofibrils isolated in borate–KCl was markedly reduced. Furthermore, for a given $MgCl_2$ concentration the inhibitory effect of ATP became apparent at lower concentrations as the ionic strength increased (Fig. 5*a*).

It was considered that exposure of the isolated myofibrils to solutions of lower ionic strength during preparation might modify their response to increasing ATP concentration. Therefore, to investigate the significance of the substrate inhibition effect under the ionic conditions prevailing *in vivo*, and in experiments with glycerated fibre models (*I*, 0·1–0·16), myofibrils were isolated in 0·1 M-KCl from fresh and glycerated psoas muscle. Fig. 5*b* shows the results obtained with myofibrils from glycerated muscle. The ATPase was much less sensitive to increasing KCl concentration—a general finding with myofibrils from both fresh and glycerated muscle which were isolated in 0·1 M-KCl. With

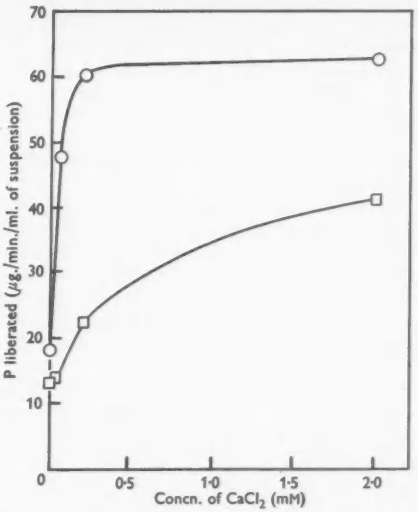

Fig. 4. Comparison of the activating effect of $CaCl_2$ on the myofibrillar ATPase in the absence and presence of $MgCl_2$. All tubes contained 7·5 mM ATP and incubations were carried out as for Fig. 1. ○, 2·5 mM-$MgCl_2$; □, $MgCl_2$ absent.

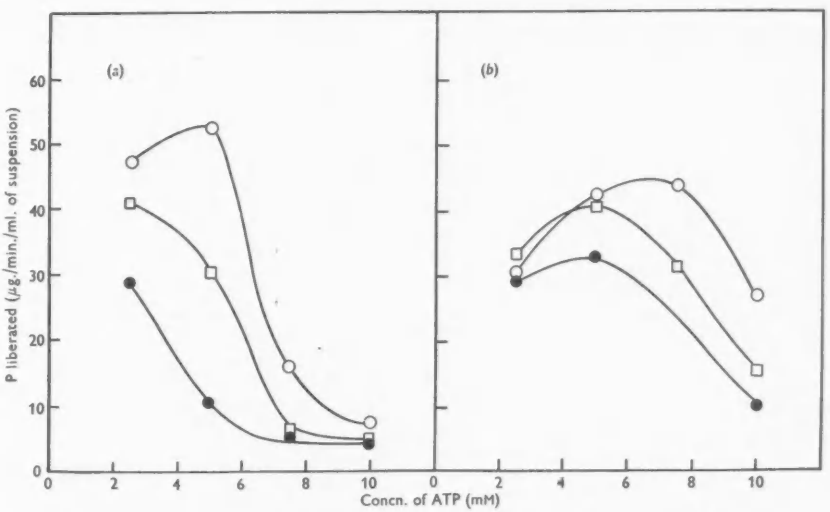

Fig. 5. Effect of ionic strength on the substrate inhibition of the myofibrillar ATPase. (*a*) Myofibrils prepared from fresh muscle in borate–KCl; (*b*) myofibrils prepared from glycerated muscle in 0·1 M-KCl. Conditions of enzyme experiments similar to those of Fig. 1, with 5 mM-$MgCl_2$ present in all cases and the following further additions: ○, no added KCl; □, 0·05 M-KCl; ●, 0·1 M-KCl.

these myofibrils the effect of increasing ATP concentration in an incubation medium of I about 0·16 is very similar to that obtained with borate–KCl prepared myofibrils in a medium of I about 0·06. At the latter ionic strength with myofibrils prepared in 0·1 M-KCl the concentration of ATP required to produce inhibition was higher than that needed at I of 0·16, and, over the range investigated, the effect obtained was much less.

It is clear that the inhibitory effect of higher substrate concentrations is shown by myofibrils isolated from fresh and glycerated muscle, but differences exist in the sensitivity of the systems to increasing ionic strength. These differences are related to the previous history of the myofibrils, in particular to the ionic conditions to which they have been exposed.

Effect of pyrophosphate

Bendall (1954) reported that inorganic pyrophosphate inhibited the magnesium-activated ATPase activity of suspensions of homogenized glycerated fibres; he suggested that the lowered rate of ATPase activity associated with relaxation could be explained by production of pyrophosphate. The inhibitory effect of pyrophosphate was investigated when myofibrils isolated from fresh muscle were used, with particular emphasis on the relative concentrations of $MgCl_2$, $Na_4P_2O_7$ and ATP. In general the $MgCl_2$ concentration was equal to, or greater than, the ATP concentration. In the former case addition of increasing amounts of pyrophosphate produced progressive inhibition, whereas in the latter case the concentration of $Na_4P_2O_7$ at which inhibition began was dependent on the concentration of $MgCl_2$ (Fig. 6). Although inhibition was not so marked as with excess of ATP, the same general relation appeared to hold in that the onset of inhibition occurred when the total pyrophosphate group concentration, i.e. ATP + inorganic pyrophosphate, exceeded the $MgCl_2$ concentration.

Effect of inosine triphosphate and other nucleotide phosphates

It was noted that certain marked differences existed between the ITPase and ATPase activity of myofibrils. With activator and substrate concentrations both equal to 5 mM, myofibrils split ATP at a high rate in the presence of $CaCl_2$ or $MgCl_2$, the activity with Mg^{2+} being somewhat lower than with Ca^{2+}. The rate of hydrolysis of ITP, however, with $MgCl_2$ was four times that with $CaCl_2$. In general, the rate of hydrolysis of ITP with magnesium was approximately one-third of that of ATP under similar conditions.

In contrast to the effect obtained with ATP (cf. Fig. 1), increasing the ITP concentration from 2 to 10 mM produced only a slight progressive fall in activity of the myofibrillar ITPase. The results illustrated in Fig. 7 were obtained when 5 mM-

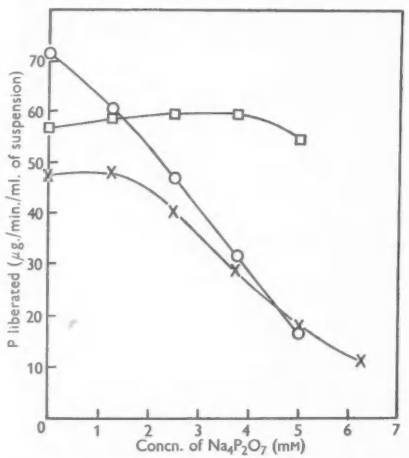

Fig. 6. Effect of $MgCl_2$ concentration on the inhibition of the myofibrillar ATPase by inorganic pyrophosphate. Additions common to all enzyme tubes were 0·05 M tris buffer, pH 7·4, and 5 mM ATP. Different myofibril preparations used for each $MgCl_2$ concentration. □, 10 mM-$MgCl_2$; ×, 7·5 mM-$MgCl_2$; ○, 5 mM-$MgCl_2$.

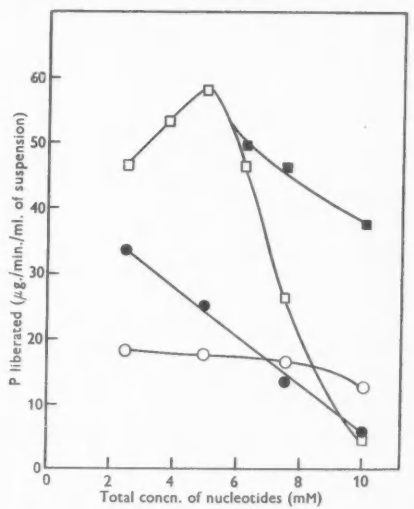

Fig. 7. Effect of substrate concentration on the splitting of ITP, UTP and ATP by myofibrils. 0·05 M tris buffer, pH 7·4, and 5 mM-$MgCl_2$ common to all incubation tubes. ○, ITP; ●, UTP; □, ATP; ■, 5 mM ATP + ADP.

$MgCl_2$ was added, but similar results were obtained with 2 and 7·5 mM-$MgCl_2$. On the other hand, when uridine triphosphatase (UTPase) activity was studied under similar conditions, increasing the substrate concentration caused a progressive increase in inhibition. The significance of this effect is not clear, as the UTP preparations contained appreciable quantities of inorganic P (equivalent to 7% of the acid-labile P) and further investigation was prevented by the shortage of UTP.

The inhibitory action of ADP was also investigated in a similar way to that employed in the experiments carried out with pyrophosphate, namely by adding ADP to a system containing $MgCl_2$ and ATP in approximately equimolar quantities. Some inhibition was obtained on increasing the ADP concentration, but this is much less than that obtained with excess of ATP (Fig. 7). In similar circumstances AMP and IMP were not inhibitory.

Effect of myokinase

Bendall (1954) has reported that, during the isolation of relaxing-factor preparations from rabbit-muscle extracts, myokinase and factor activity are closely correlated at all stages of the purification. These preparations also inhibited the ATPase activity of homogenates of glycerated fibres, and Bendall's results suggest that under certain conditions myokinase itself can inhibit the myofibrillar ATPase.

At pH 7·4 in tris buffer, added myokinase had no significant effect on the initial rate of ATPase activity of washed myofibrils. Varying amounts of enzyme were used in a system containing 5 mM-$MgCl_2$ and 5 mM ATP, and the ATP concentration was varied between 2 and 10 mM in the presence of 2, 5 and 10 mM-$MgCl_2$. On addition of 0·05 and 0·10 M-KCl to a system containing 5 mM-$MgCl_2$ and 5 mM ATP, myokinase likewise failed to produce inhibition of the ATPase.

This lack of effect cannot be attributed to the myokinase in the myofibril suspensions, since they probably contain very much less than glycerated fibres with which the inhibitory effect of myokinase was demonstrated by Bendall (1954).

Effect of ethylenediaminetetraacetate

Recently several studies (Friess, 1954; Friess, Morales & Bowen, 1954; Bowen & Kerwin, 1954) on the effect of EDTA on myosin ATPase have been reported. For the most part the conditions of study do not readily allow us to relate these results to the investigations (Bozler, 1954; Watanabe, 1955) which have been carried out on the relaxing action of this chelating agent on the glycerated-fibre model. Some experiments on the effect of EDTA on the myofibrillar ATPase are reported here, as they bear particularly on this problem and the role of magnesium in the relaxation process. They form part of a general study of EDTA on the interaction of ATP, actin and myosin which is now in progress and which has been reported (Perry & Grey, 1956).

Fig. 8 shows the effect of the addition of increasing amounts of EDTA to the myofibrillar ATPase system activated by $MgCl_2$ or $CaCl_2$. In the presence of $MgCl_2$, and when the concentration of the chelating agent was 0·2–0·5 mM, pronounced inhibition, largely independent of the $MgCl_2$ concentration, was apparent. For example 0·5 mM EDTA produced 75–80% inhibition, whether the $MgCl_2$ concentration was 1 or 10 mM. On the other hand, much higher concentrations of EDTA were required to produce similar degrees of inhibition of the calcium-activated ATPase under otherwise similar conditions. In the latter case the quantitative aspects of the inhibition were such to suggest that it was caused by the removal from the system of the major part of the added calcium as the EDTA complex. It does not seem possible to explain the results with $MgCl_2$ on a similar basis, however, for EDTA has a stronger affinity for Ca^{2+} than for Mg^{2+} ions (Calvin, 1954), and is strongly inhibitory when the amount of EDTA present is such that only a very small fraction of the Mg^{2+} ions would be expected to be bound. These results with magnesium may explain Watanabe's (1955) finding that EDTA induced relaxation of glycerated fibres when present

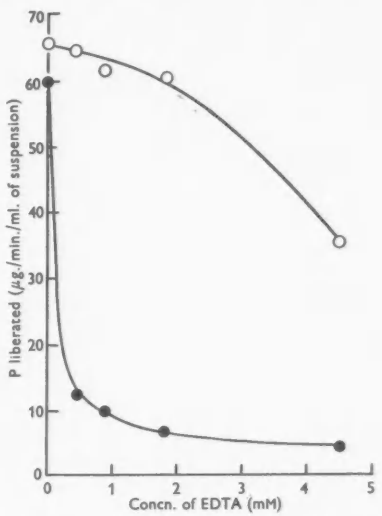

Fig. 8. Effect of EDTA on the Ca- and Mg-activated ATPase of myofibrils. Incubations carried out for 5 min. at 20·5° in the presence of 0·05 M tris buffer, pH 7·5, 5 mM-ATP. ○, 5 mM-$CaCl_2$; ●, 5 mM-$MgCl_2$.

in one-tenth of the concentration of the $MgCl_2$, for although only a small fraction of the magnesium in the system would be present as a complex under these conditions, the ATPase activity of the myofibrils will be low.

DISCUSSION

With the exception of myokinase all the substances that are known to produce relaxation in the glycerated-fibre models studied in this investigation have been shown to bring about a marked inhibition of the magnesium-activated ATPase of isolated myofibrils. Although the results obtained with myokinase do not confirm those obtained by Bendall (1954) with glycerated-fibre preparations, they are in agreement with the recent findings of Kumagai, Ebashi & Takeda (1955), who have provided evidence that myokinase alone was not adequate to produce relaxation in well-washed fibres.

The inhibition obtained with either ATP or $Na_4P_2O_7$ is clearly dependent on the magnesium concentration, and the effect at low ionic strength produced by excess of substrate on myofibrils isolated in borate–KCl resembles in a striking way the effect on pyrophosphatase (cf. Bauer, 1937; Bailey & Webb, 1944; Heppel & Hilmoe, 1951; Gilmour & Calaby, 1953; McElroy, Coulombre & Hays, 1951; Bloch-Frankenthal, 1954; Robbins, Stulberg & Boyer, 1955). The last two groups of workers have both concluded, from analysis of their data, that the substrate for the pyrophosphatase enzyme is $(MgP_2O_7)^{2-}$ and that free inorganic pyrophosphate inhibits the enzyme. Superficially the similarities between the pyrophosphatase and myofibrillar ATPase systems are sufficiently close to suggest that a similar mechanism may exist when magnesium is the activating cation for the ATPase, i.e. that Mg-ATP is the true substrate, whereas free ATP is not split but is bound at the enzymically active sites of the actomyosin system and causes inhibition.

It should be emphasized, however, that such a function of magnesium would appear to be confined to the myofibrillar actomyosin system, since the ATPase of myosin is not activated by magnesium. Both calcium and magnesium activate the myofibrillar ATPase, but the myosin ATPase requires calcium and it is clear that considerable differences exist in the characteristics of ATP hydrolysis by the myofibril in the presence of these two metals. Earlier work (Chappell & Perry, 1955) has shown that the stimulation of myofibrillar ATPase activity by magnesium disappears at 0°, and at higher temperatures as the ionic strength increases (Perry, 1951). The present study provides further examples of differences, in that, with calcium as activator, substrate inhibition is not obtained over the range at which this phenomenon is obtained with magnesium, and that the magnesium-activated myofibrillar ATPase is very much more sensitive to EDTA. It is possible that the difference in the effect of excess of substrate on activity of the myofibrils in the presence of calcium and magnesium reflects differences in the complex-forming properties of these metals with ATP rather than in fundamental mechanism. From this point of view it may be significant that, whereas with 5 mM ATP optimum activation is obtained with 5 mM-$MgCl_2$, with $CaCl_2$ the optimum concentration is much higher than the ATP concentration.

The results obtained when inorganic pyrophosphate is introduced into the ATPase system can be explained by assuming either that free inorganic pyrophosphate inhibits the hydrolysis of the substrate, Mg-ATP, or that the inorganic pyrophosphate preferentially binds Mg^{2+} ions, leaving ATP in excess to produce inhibition. $(MgP_2O_7)^{2-}$ ions do not inhibit the enzyme, since if there is enough magnesium to combine with both ATP and inorganic pyrophosphate the high level of activity is maintained. It is well known that ATP (Hers, 1952) and inorganic pyrophosphate (Rogers & Reynolds, 1949) form complexes with Mg^{2+} ions, but as adequate information is not available to assess the relative affinities of these two substances for the metal under the conditions of the enzyme experiments, it is not possible to decide which of these two alternatives is more likely.

ITP and UTP differ in behaviour from ATP in that a progressive fall in activity was obtained with increasing UTP concentration, whereas little inhibition was obtained when ITP was used as substrate. If the mechanism for the hydrolysis of ITP in the presence of magnesium is similar to that suggested above for ATP, these results would imply that free ITP has little affinity for the active centres of the myofibrillar ATPase. From this point of view the absence of the amino group in the 6-position of the purine ring may be a relevant factor.

Most of the studies described have been carried out with myofibrils isolated in borate–KCl, the magnesium-activated ATPase of which appears to be particularly sensitive to an increase in the ionic strength. Nevertheless, the results can be related to events occurring *in situ* where the ionic strength is about 0·16, for myofibrils freshly isolated in 0·1 M-KCl from both fresh and glycerated muscle, which have been subsequently studied in an incubation medium of I approx. 0·16, behave with respect to increasing ATP concentration very much like borate–KCl myofibrils at I approx. 0·06. It appears that, after isolation of myofibrils and subsequent storage in the borate–KCl medium, the magnesium-activated ATPase becomes more sensitive to the inhibitory effects of higher ionic strength.

With both preparations, however, the level of ATP which is inhibitory at a given $MgCl_2$ concentration falls as the ionic strength is increased. With fructokinase, Hers (1952) found that the optimum ATP:Mg ratio fell from 2 to 1 with increasing ionic strength.

Hasselbach (1952) has reported that the addition of 0·1 mM-$MgCl_2$ lowers the concentration at which ATP begins to inhibit the ATPase activity of actomyosin gels. These results appear to be contrary to those reported here, but they are perhaps not strictly comparable, as in all cases the ATP concentration (1–10 mM) was much greater than that of the $MgCl_2$.

It seems likely that the low rate of hydrolysis of ATP characteristic of the myofibril in relaxation could be due to the presence of ATP in excess of the available magnesium in the muscle. Strong suggestion that substrate inhibition of the type described above occurs in relaxation (at least in the fibre models) is given by the fact that in the presence of calcium, which relieves the inhibition of the myofibrillar ATPase, it is impossible to make glycerated fibrils relax (Bendall, 1953*a*). Furthermore, the failure of Hasselbach (see Weber, 1955) to demonstrate relaxation with ITP in the presence of the relaxing factor could be explained by the finding that the substrate inhibition effect was not obtained when this nucleotide was split by myofibrils. The relative concentrations of magnesium and ATP in muscle are such that if all the magnesium present were available to the myofibrillar ATPase system, then the enzymic activity would be high. It has been suggested (Perry, 1955, 1956) that in relaxed muscle most of the magnesium is bound to the relaxing factor to ensure that the ATP concentration is in the relative excess necessary to produce inhibition. This view is supported by the experiments with inorganic pyrophosphate, the relaxing actions of which can be explained on the basis that it forms a complex with the magnesium with the result that the concentration of the available magnesium is exceeded by that of the ATP. It appears that with EDTA, however, inhibition is obtained when only a very small fraction of the magnesium would be bound as a complex and when its level would not be reduced sufficiently to produce any appreciable inhibition due to excess of ATP. It is possible that the EDTA complex is not with magnesium but with small amounts of some other cation present in the system which is essential for magnesium activation. The results with EDTA support the general hypothesis that relaxation is associated with a low rate of ATP hydrolysis, but the fact that this is achieved when the amount of EDTA present is adequate to form a complex with only a small fraction of the magnesium present reflects some unique aspect of the mechanism of ATPase activity of the myofibril.

The studies with isolated myofibrils indicate that, with certain combinations of ionic strength, substrate concentration and activator concentration which are comparable to those occurring in the living muscle, it is possible to obtain, in the absence of the recognized relaxing factors, the low levels of ATPase activity characteristic of relaxation. In previous investigations with glycerated-fibre models the importance of the relative concentrations of ATP and magnesium has not been recognized, although Bendall (1953*a*) noted that the magnesium concentration necessary for the demonstration of contraction was very critical.

If it is accepted that low enzymic activity, together with the presence of a plasticizer to reduce the interaction of actin and myosin, are the requirements of relaxation, then on the basis of the findings reported in this paper relaxing factors can be divided into two classes: (1) those which bind metal activators, e.g. (*a*) inorganic pyrophosphate, which binds magnesium, and (*b*) EDTA (the effect of EDTA is exceptional in that it produces marked inhibition of magnesium-activated ATPase at concentrations that are sufficient to bind only a small fraction of the total magnesium); (2) those substances or systems which act by maintaining the ATP at the inhibitory level, e.g. creatine phosphokinase, and perhaps myokinase.

It appears that the Marsh factor acts by binding a metal activator, but there is not yet enough evidence to decide whether this factor is a specific protein or whether it is some protein or enzyme system already recognized in muscle.

SUMMARY

1. At ionic strength about 0·06 and pH 6·9–7·6 the concentration at which adenosine triphosphate (ATP) became inhibitory for the adenosine triphosphatase (ATPase) of myofibrils isolated in 0·039 M borate buffer (pH 7·1)–0·025 M-KCl, was dependent on the concentration of magnesium chloride added to the system. At 5 mM-$MgCl_2$ inhibition occurred when the molar concentration of ATP exceeded that of magnesium.

2. The substrate inhibition of the magnesium-activated ATPase did not occur in the presence of calcium chloride in concentrations one-tenth to one-twentieth of that of the magnesium chloride.

3. Myofibrils prepared in 0·1 M-KCl from fresh and glycerated muscle exhibited similar substrate inhibition effects at I about 0·16 to those obtained at I about 0·06 with myofibrils isolated in borate–potassium chloride.

4. Inorganic pyrophosphate inhibited the magnesium-activated myofibrillar ATPase when the total concentration of ATP and pyrophosphate exceeded that of magnesium chloride.

5. Adenosine diphosphate (ADP) was less inhibitory than ATP when it replaced part of the latter; inosine triphosphate had little inhibitory effect. Over a similar range, increasing concentration of uridine triphosphate produced progressively increasing inhibition.

6. No inhibitory action on the magnesium-activated myofibrillar ATPase could be demonstrated with myokinase preparations.

7. Ethylenediaminetetraacetate strongly inhibited the magnesium-activated myofibrillar ATPase in concentrations which were only one-twentieth of that of the added magnesium chloride. Calcium activation of the ATPase was much less sensitive to ethylenediaminetetraacetate.

8. These findings are discussed in relation to the relaxation studies by other workers on glycerated muscle fibres.

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The Phospholipase *B* of Liver

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Previous isotopic experiments have indicated that the free glycerylphosphorylcholine (GPC) and glycerylphosphorylethanolamine (GPE) present in liver tissue are intermediates in the metabolic breakdown of phosphatidylcholine and phosphatidylethanolamine respectively (Dawson, 1955). In support of this conclusion an enzyme, GPC diesterase, has been found in liver tissue which specifically hydrolyses GPC and GPE into glycerophosphoric acid and the free base (Dawson, 1956*a*). The present paper reports some observations on a phospholipase *B* (nomenclature of Zeller, 1952) which occurs in liver, forming GPC and probably GPE by the splitting of the single saturated fatty acid from lysolecithin or lysophosphatidylethanolamine.

The enzyme phospholipase *B* has a comparatively wide distribution in nature. Contardi & Ercoli (1933), studying rice bran, were probably the first to recognize that it was a specific enzyme which did not attack lecithin until one fatty acid had been removed by phospholipase *A*. The enzyme has also been found to occur in moulds (Fairbairn, 1948) and bacteria (Hayaishi & Kornberg, 1954). Schmidt, Hershman & Thannhauser (1954) and Schmidt, Greenbaum, Fallot, Walker & Thannhauser (1955) observed the accumulation of the ethanol-soluble GPC in autolysing pancreas and intestine, but not

in liver. Consistent with this is the demonstration by Shapiro (1953) of an active phospholipase *B* in glycerinated extracts of ox pancreas. The properties of the phospholipase *B* of liver were found to resemble most closely those of this pancreatic enzyme.

EXPERIMENTAL

Enzyme preparation

Phospholipase *B* activity was mainly studied in extracts from acetone-dried rat liver, which was prepared by a method previously described (Dawson, 1956*a*). The acetone-dried liver (1 g.) was extracted first with 7·5 ml. of 50% (v/v) aqueous glycerol, to which was then added 7·5 ml. of 0·1% $NaHCO_3$ according to the method of Shapiro (1953). After centrifuging at 10 000 *g* for 20–30 min., the slightly turbid supernatant was removed and either used for experiments directly or dialysed overnight at 0° against a solution of 25% (v/v) glycerol and 0·05% $NaHCO_3$. The extracts only slowly lost activity when stored at −20°.

Materials

Ovolecithin was prepared by the method of Hanahan (1954), in which the crude phospholipids are fractionated on an alumina column. Lysolecithin was prepared enzymically by the action of cobra (*Naja naja*) venom on lecithin dissolved in diethyl ether according to the general directions of Hanahan (1954) and Hanahan, Rodbell & Turner (1954). The gelatinous precipitate which formed was evaporated almost to dryness in a rotary drier *in vacuo*, and then dissolved in the minimum volume of $CHCl_3$. The lysolecithin was precipitated by the addition of 10 vol. of ether and recovered by centrifuging. The precipitation was repeated twice, and the lysolecithin stored in $CHCl_3$ solution which was centrifuged or filtered to remove any turbidity. It had a fatty acid:P ratio of 1·05:1, and was completely water-soluble. According to Hanahan (1954) the fatty acid remaining after the attack of venom on ovolecithin is on the β-carbon atom, and consists of stearic acid, although Levene, Rolf & Simms (1924) and Rhodes (private communication) have found that other saturated fatty acids are also present.

L-α-GPC was prepared by a previously described method (Dawson, 1956*a*). Lysophosphatidylethanolamine was a gift from Dr D. N. Rhodes. Diphenylchloroarsine and bromoacetophenone were kindly supplied by Sir Rudolph Peters, F.R.S., and the *Naja naja* (common Indian cobra) venom by the Zoological Society of London.

Measurement of GPC liberation

The activity of the enzyme was usually measured by estimating the GPC liberated during the incubation. The usual incubation medium consisted of 0·25 ml. of Na_2HPO_4–KH_2PO_4 buffer (0·3 M), pH 6·4, 0·2 ml. of 50% (v/v) glycerol, 0·2 ml. of the enzyme preparation, and 0·1 ml. of lysolecithin (0·03 M). After incubation at 37° the reaction mixture was placed in ice and 0·25 ml. water added, followed by 0·15 ml. of 5% serum albumin (Armour) solution to assist in the precipitation of lysolecithin. The mixture was treated with 0·5 ml. of 10% trichloroacetic acid solution, and centrifuged. A 1·1 ml. sample of the supernatant was mixed with 0·25 vol. of 5 N-HCl and hydrolysed at 100° for 20 min. to hydrolyse the GPC. The hydrolysate was centrifuged and a 1·0 ml. sample removed from the precipitate which developed during the hydrolysis. Choline was then estimated by the procedure of Appleton, La Du, Levy, Steel & Brodie (1953), 0·3 ml. of the cold triiodide reagent being added directly to the hydrolysate. It was found necessary to keep all solutions in ice throughout the estimation. The choline periodide was made up in ethylene dichloride to a final volume of 10 ml. On reading at 365 mμ., 120 μg. of GPC gave an optical density of approximately 0·64. It was observed that the recovery of GPC added to reaction mixtures tended to be low, especially with small concentrations of GPC. Moreover, the lysolecithin substrate blanks were, although constant, reasonably high, presumably due to the incomplete precipitation of the lysolecithin by the trichloroacetic acid, resulting in its hydrolysis to free choline and estimation as GPC. Fortunately, however, the recovery of GPC from reaction mixtures containing lysolecithin was linear between 0 and 240 μg. Consequently, by adding lysolecithin after the incubation to the tubes to which it had not previously been added, it was possible to obtain a reasonable estimate of the GPC produced by the enzymic action. It was observed that many other compounds, e.g. adenosine triphosphate (ATP) and eserine, when present in the incubation medium gave precipitates with iodine which absorbed at 365 mμ., and consequently careful control experiments were necessary.

Free fatty acid estimation

The reaction mixture (0·75 ml.) was treated with an equal volume of ethanol and then with 0·1 ml. of 5 N-HCl. The mixture was shaken mechanically for 5 min. with 3 ml. light petroleum (b.p. 60–80°), centrifuged, and 2 ml. of the upper layer carefully withdrawn without touching the walls of the tube. To the sample was added 3 ml. of acetone, followed by 0·15 ml. of 0·04% cresol red indicator. The fatty acid was then titrated with 0·01 N-KOH in methanol using a stream of N_2 to stir the solution. Recovery of a stearic acid standard added to the reaction mixture was linear between 0 and 5 μmoles, the maximum tested. Failure to add ethanol resulted in practically no extraction of fatty acid from the reaction mixture. The method is a far less sensitive measure of phospholipase *B* activity than the estimation of GPC liberation, a titration difference of 0·1 ml. corresponding to a breakdown of 1 μmole of substrate.

Miscellaneous estimations

In a few experiments, disappearance of acyl ester in the reaction mixture was measured by a modified hydroxamic acid method (Stern & Shapiro, 1953).

The liberation of water-soluble amino N from lysophosphatidylethanolamine was measured by using the Moore & Stein (1948) ninhydrin colorimetric method, after the reaction mixture had been treated with serum albumin and trichloroacetic acid as for the GPC estimation. The trichloroacetic acid in the supernatant was removed by ether extraction before the estimation. In all these experiments the enzyme was dialysed to reduce its free amino N content. Acid-soluble phosphorus was estimated by the procedure of Fiske & Subbarow (1925).

RESULTS

Although the conditions of the enzyme preparation were standardized as far as possible, the preparations from individual animals showed considerable

variation in activity. However, activity was always detectable, and at a substrate level of 4×10^{-3} M, 10–30 % of the lysolecithin was broken down in the first hour. During this time the production of GPC in a standard reaction mixture occurred at a constant rate, suggesting that during this period the enzyme was not inhibited by its reaction products. Dialysis of the enzyme preparation against 25 % (v/v) glycerol–0·05 % (w/v) $NaHCO_3$ overnight at 0° resulted in a loss of some activity (35 %), but dialysis overnight against water resulted in an even greater loss of activity (80 %). The activity of the enzyme preparation dialysed against water was not improved by the addition of glycerol to the incubation medium. The stabilizing effect of glycerol on enzyme preparations is a well-recognized phenomenon (Bayliss, 1919; Krebs & Eggleston, 1944; Fairbairn, 1948; Shapiro, 1953).

Nature of the enzyme-catalysed reaction

The acid-labile choline liberated during activity was associated with the production of an equivalent amount of acid-soluble phosphorus. This must represent GPC, as phosphorylcholine is very stable to acid hydrolysis (Plimmer & Burch, 1937). No free choline could be detected among the reaction products at pH 6·4, presumably because at this pH GPC-diesterase activity of the extract would be negligible (Dawson, 1956*a*). The liberation of acid-labile choline was associated with the formation of free fatty acid and a decrease of acyl ester bonds. These observations are consistent with phospholipase *B* activity, i.e. lysolecithin + H_2O → GPC + fatty acid.

Effect of substrate concentration

An example of the variation of enzyme activity with the concentration of lysolecithin in the incubation media is shown in Fig. 1. In three enzyme preparations tested, maximal activity was observed with substrate levels between 2 and $3{\cdot}5 \times 10^{-3}$ M. Activity was reduced at higher concentrations of lysolecithin. A similar inhibition at high substrate concentrations has been observed for the phospholipase *B* of pancreas (Shapiro, 1953).

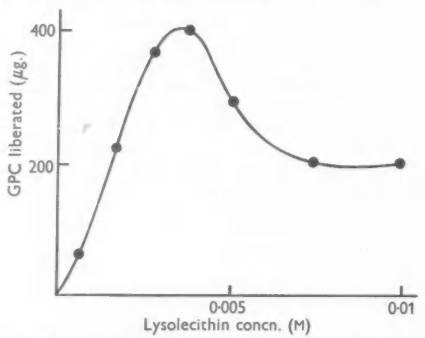

Fig. 1. Effect of substrate concentration on the hydrolysis of lysolecithin by liver phospholipase *B*.

pH optimum

Maximal activity of the liver enzyme, measured in a series of phosphate buffers, was observed at about pH 6·2 (Fig. 2). This is similar to the optimum activity of ox-pancreatic enzyme (pH 6·0) (Shapiro, 1953), but distinct from that of phospholipase *B* in *Penicillium notatum* (pH 3·8–4·4) (Fairbairn, 1948), and that for the corresponding enzyme of *Aspergillus oryzae* (pH 3·5) (Contardi & Ercoli, 1933).

Effect of inhibitors, metals, etc.

With a few preparations a small stimulation of the activity was observed on the addition of cysteine (10^{-2} M). The effect, however, could not be made reproducible, and in fact most preparations showed

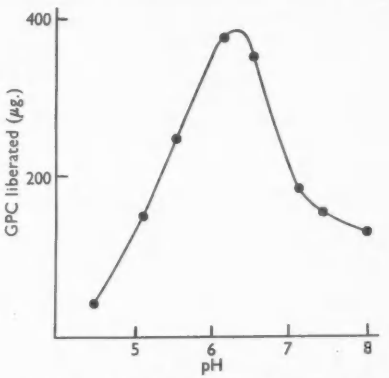

Fig. 2. Variation of liver phospholipase *B* activity with pH. Value at pH 4·4 obtained in sodium acetate–acetic acid buffer, other activities in Na_2HPO_4–KH_2PO_4 buffers.

Table 1. *Effect of substances reacting with sulphydryl groups on liver phospholipase* B *activity*

Inhibitor	Concn. (mM)	Inhibition (%)
Hg^{2+}	0·1	37
	1·0	94
Cu^{2+}	0·1	38
	1·0	75
Zn^{2+}	1·33	74, 65
p-Chloromercuribenzoic acid	0·1	2, 4
Iodoacetic acid	5·0	2, 0
Bromoacetophenone	5·0	40*, 38*
Diphenylchloroarsine	0·5	42, 46*

* Pre-incubated 5 min. with enzyme preparation.

negligible cysteine activation. The effect of various substances reacting with sulphydryl groups is given in Table 1. Although some of these inhibitors were active against the enzyme, the high concentrations needed suggest that any sulphydryl groups on the enzyme play only a minor role in the formation of the active centre. Fairbairn (1948) reported that mould phospholipase *B* was inhibited by copper, but its sensitivity appears to be much less than that of the liver enzyme. The mould enzyme is also different in its sensitivity to cyanide; whereas liver phospholipase *B* is not inhibited by 0·01 M cyanide, suppression of the mould enzyme is virtually complete (97 %) (Fairbairn, 1948).

Calcium, magnesium, manganese and ferrous ions all produced a slight inhibition of the enzyme when added at a concentration of $1{\cdot}33 \times 10^{-3}$ M. Three enzyme preparations tested in the presence of 10^{-3} M ethylenediaminetetraacetic acid all showed a stimulation of activity (13, 73, 4 %) which is probably dependent upon the removal of contaminating traces of heavy metals. It confirms that the enzyme activity is not dependent on the presence of bivalent metal ions, e.g. Ca^{2+} (cf. Contardi & Ercoli, 1933).

Fluoride (0·02 M) produced a small inhibition of activity (34 %); histidine (5×10^{-3} M), di*iso*propyl phosphorofluoridate ($1{\cdot}33 \times 10^{-5}$ M), eserine (2×10^{-4} M) and 2-acetamido-1:3:4-thiadiazole-5-sulphonamide (10^{-4} M) did not diminish the activity. Francoli (1937) reported the almost complete inhibition of phospholipase *B* in wasp venom by eserine at about one-third of the concentration used in the present experiments. Saturation of the incubation medium with diethyl ether, or the addition of 6 % (v/v) ethanol, gave large inhibitions of activity. This is in contrast to the behaviour of phospholipases *A*, *C* and *D*, where activity appears to be enhanced by the presence of ether (Hanahan, 1952; Hanahan *et al.* 1954; Kates, 1954).

When the acetone-dried liver extract (pH 7·1) was kept at 55° for 10 min. activity was virtually abolished, whereas when kept at 50° for 10 min. there was no appreciable diminution of activity. Noguchi (1944) has observed that a preparation from lung containing phospholipase *B* was inactivated at 60°. This heat lability of phospholipase *B* may be contrasted with the outstanding heat resistance of phospholipase *A*.

Specificity

When the enzyme preparation was incubated with emulsions of lecithin for long periods at varying pH values it was found that no liberation of fatty acid or decrease of acyl ester bonds occurred. Furthermore, on incubating lecithin with the enzyme preparation at pH 6·4, no liberation of GPC occurred, although the presence of small quantities of lysolecithin added to the system could readily be detected by this method.

The preparation possessed a small amount of lipase activity as measured with triolein as substrate, but this was readily distinguished from phospholipase *B* by its complete thermostability on keeping at 55° for 10 min.

When the enzyme preparation was incubated with a sample of lysophosphatidylethanolamine for 90 min. at varying pH values no liberation of water-soluble amino N or P was detectable. However, it was thought that this lack of activity might be due to the insolubility of the lysophosphatidylethanolamine in the aqueous medium. Consequently, a sample of mixed lysophosphoglycerides was prepared by allowing *Naja naja* venom to act on a crude egg-phospholipid preparation dissolved in ether. The ether-insoluble material was purified in the same way as has been described for the preparation of lysolecithin. This material gave a clear solution in water, and on incubation with a dialysed enzyme preparation there was a slow liberation of acid-soluble amino N. It was also found that mixtures of lysophosphatidylethanolamine and lysolecithin (1:2·75 mol.prop.) were soluble in water, and again on incubation with the dialysed enzyme acid-soluble amino N was produced. It can be concluded, therefore, that the liver extract will attack lysophosphatidylethanolamine as well as lysolecithin, although it cannot be assumed that the same enzyme is responsible. However, it is significant that added lysophosphatidylethanolamine will inhibit lysolecithin breakdown.

Distribution of the enzyme in animal tissues

Appreciable liberation of GPC was observed when glycerinated extracts of acetone-dried rat kidney, spleen, brain and blood were incubated with lysolecithin at pH 6·4. Little activity could be detected in similar extracts from heart or skeletal muscle. Phospholipase *B* was also found in sheep liver, kidney and thyroid, but very little activity was detectable in whole blood.

DISCUSSION

The present report of an active phospholipase *B* in rat liver adds to the evidence already obtained that the pathway of phosphatidylcholine and phosphatidylethanolamine catabolism in this tissue is via the glycerylphosphoryl esters (GPC and GPE) (Dawson, 1955, 1956*a*). Of the other possible routes for the breakdown of these phosphoglycerides in the tissue, it has been shown isotopically that the large amounts of phosphorylcholine present in the liver are not formed by phospholipase *C* activity (Dawson, 1956*b*), nor has it been possible to obtain in our experiments any evidence for the presence of

a phospholipase *D*. Consequently, the 'glyceryl-phosphorylester intermediate' route probably represents the major if not the sole pathway of phosphoglyceride catabolism in the liver.

Although lysolecithin would be an intermediate in the route

$$\text{lecithin} \xrightarrow{\text{phospholipase } A} \text{lysolecithin} \xrightarrow{\text{phospholipase } B} \text{GPC} \xrightarrow{\text{GPC diesterase}} \text{glycerophosphoric acid} + \text{choline},$$

its presence has never been demonstrated with certainty in fresh mammalian tissues. However, an accumulation of this intermediate may be undesirable for the animal because of its well-known property of disrupting cell membranes. Consequently, an active phospholipase *B* would be a necessity, especially in tissues which can rapidly catabolize phosphoglycerides.

SUMMARY

1. The presence of an active phospholipase *B* has been demonstrated in glycerinated extracts of acetone-dried rat and sheep liver, with lysolecithin as a substrate.

2. The liver extracts can also break down lysophosphatidylethanolamine when this is added in a soluble form.

3. Maximal hydrolysis of lysolecithin was obtained at pH 6·2 and a substrate concentration between 2 and $3{\cdot}5 \times 10^{-3}$ M.

4. The enzyme is resistant to most enzyme inhibitors. However, Hg^{2+}, Cu^{2+} and Zn^{2+} ions are inhibitory, as is saturation of the incubation medium with ether.

5. Lysolecithin breakdown has also been observed to occur with extracts from acetone-dried rat kidney, brain, heart, spleen and whole blood, but not with skeletal muscle. Phospholipase *B* is also present in sheep kidney and thyroid.

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Protection of Succinic Dehydrogenase Thiol Groups by Fluoride and Phosphate

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The biological oxidation of succinate is inhibited by fluoride (Batelli & Stern, 1911). Several explanations have been proposed for fluoride action, e.g. competition of fluoride with succinate for succinic dehydrogenase [D. J. O'Kane, unpublished data quoted by Potter & Schneider (1942)]. Phosphate is necessary for the inhibitory action of fluoride (Slater & Bonner, 1952), and kinetic deductions led these authors to postulate the formation of a slightly dissociated inactive complex made up of fluoride, dehydrogenase and phosphate in which fluoride and phosphate compete with succinate for the enzyme active centre.

Succinic dehydrogenase is protected from thiol reagents by succinate and competitors of succinate, e.g. malonate (Hopkins, Morgan & Lutwak-Mann, 1938; Potter & DuBois, 1943; Barron & Singer, 1945), oxaloacetate, suramin and 1-naphthylamine

4:6:8-trisulphonate (Stoppani & Brignone, in preparation). A similar protection of the enzyme by the joint action of fluoride and phosphate would support the theory of the fluoride–enzyme–phosphate complex.

EXPERIMENTAL METHODS

Succinoxidase. This enzyme was obtained from pig-heart muscle as described by Bonner (1954). The acid precipitation took place at 4° and the precipitate was spun off at 2° and 2500 rev./min. in an International Refrigerated PR 2 centrifuge. The enzyme was suspended in five times its volume of borate buffer (Palitzsch's buffer, as described by Clark, 1928), 0·1M, pH 7·3, and recentrifuged for 30 min. at 13 000 rev./min. in a Servall SS1 centrifuge. The succinoxidase was then suspended in borate buffer (0·2M) at pH 7·3. In this manner, the phosphate remaining from the heart-muscle initial extraction was greatly reduced. The residual free phosphate present in each succinoxidase preparation was estimated according to Fiske & Subbarow (1925) in the supernatant after precipitation of succinoxidase with 3·3% (w/v) trichloroacetic acid (first step in measuring the dry weight of succinoxidase, according to Slater, 1949*a*).

Enzyme activities. Succinic dehydrogenase and succinoxidase were measured manometrically at 37°, as described by Slater (1949*a*).

General procedure. Unless stated otherwise, the succinoxidase suspensions (0·8–0·9 ml.) were made up to 1·0 ml. with 0·1–0·2 ml. of ion mixture and 0·01–0·05 ml. of thiol reagent solution (final pH, 7·3). After standing at 37° the required time, the enzyme suspensions were diluted with phosphate buffer or distilled water and 0·3 ml. portions placed in the side bulb of Warburg vessels which in their main compartment contained the reagents necessary to measure succinic dehydrogenase. After 10 min. equilibration, enzyme activities were recorded. Controls not treated with sulphydryl reagents or ion mixtures were measured simultaneously. Succinoxidase inactivation with BAL was carried out as described by Slater (1949*b*).

Reagents. The following were used: iodoacetic acid and ethylenediaminetetraacetate (disodium salt, pure) from British Drug Houses Ltd.; *o*-iodosobenzoic acid, prepared according to Askenasy & Meyer (1893); 3-amino-4-hydroxyphenylarsenoxide hydrochloride (mapharside), from Parke Davis and Co.; 3-melaminylphenylarsenoxide (melarsen oxide), from May and Baker Ltd.; *N*-ethylmaleimide, from Schwarz Laboratories, Inc.; 2:3-dimercaptopropanol (BAL), from Boots Pure Drug Co. Other reagents were of analytical grade.

To avoid the possible presence of pyrophosphate in phosphate buffers these were prepared by adjusting to pH 7·32 with NaOH–10 % (w/v) phosphoric acid boiled under reflux for 10 hr. The final concentration of phosphate in these buffers was measured according to Fiske & Subbarow (1925).

Expression of results. The amount of succinoxidase used in the experiments is represented by the weight (mg.) of the protein defatted and dried according to Slater (1949*a*).

The effect of fluoride and other ions on the inactivation of succinic dehydrogenase by sulphydryl reagents has been calculated from equation (1), where P is the protection (%) of succinic dehydrogenase, I_{tr} the inhibition (%) of succinic dehydrogenase by the thiol reagent, and $I_{i,\,tr}$ the relative diminution (%) of succinic-dehydrogenase activity in the presence of thiol reagent, fluoride and/or other ions. All inhibitions have been calculated in relation to the respective controls:

$$P = \{I_{tr} - I_{i,\,tr}\}/I_{tr} \qquad (1)$$

RESULTS

Effect of fluoride and phosphate on the action of thiol reagents. As shown in Table 1, succinic dehydrogenase is inactivated by arsenicals, *o*-iodosobenzoate, iodoacetate and *N*-ethylmaleimide. This is consistent with previous observations by Hopkins & Morgan (1938), Potter & DuBois (1943), Barron & Singer (1945) and Slater (1949*c*). The effect of *N*-ethylmaleimide, which reacts quantitatively with thiols (Tsao & Bailey, 1953), could be inferred from that of maleate, also an inhibitor of succinic dehydrogenase (Morgan & Friedmann, 1938).

The inactivation of succinic dehydrogenase by mapharside and by the lower concentrations of *o*-iodosobenzoate and *N*-ethylmaleimide, is decreased in the presence of phosphate. Fluoride diminishes slightly the action of mapharside, melarsen oxide and the lower concentration of *o*-iodosobenzoate. It is difficult to decide whether this action of fluoride is dependent on the small concentration of residual phosphate of the succinoxidase preparations.

Fluoride and phosphate produce together a remarkable diminution in the action of all the sulphydryl-binding compounds tested. When there is protection of succinic dehydrogenase by either fluoride or phosphate, the protection obtained in association always greatly exceeds the numerical addition of their individual actions. At a fixed concentration of phosphate, the protection given to succinic dehydrogenase increases when the amount of fluoride is increased, whereas with fluoride concentration constant the protection increases with the amount of phosphate (Table 2). This recalls the experiments on succinic-dehydrogenase inhibition by fluoride and phosphate reported by Slater & Bonner (1952).

Specificity of fluoride action. The ability of fluoride to protect succinic dehydrogenase in the presence of phosphate seems to be unique. Table 3 shows that equal concentrations of chloride and bromide rather increase the effect of thiol reagents on the enzyme; iodide has a slight protecting action and thiocyanate scarcely affects the extent of the inactivation. With the lower concentration of phosphate (due to the residual phosphate in succinoxidase) the halogen ions exert a very weak protection on the enzyme with the exception, however, of chloride.

Table 1. *Protection of succinic dehydrogenase by fluoride in the presence of phosphate*

Succinoxidase (1·0 ml.) treated with inhibitors and additions as shown was incubated at 37° for 40 min. (for melarsen oxide) or 30 min. (for other reagents) and was then diluted with 4·0 ml. of 0·4 M phosphate, pH 7·3, or 5·0 ml. of distilled water (for melarsen oxide). Succinic-dehydrogenase activity was measured in 0·3 ml. of this extract.

Succinoxidase (mg./ml.)	Residual phosphate of succinoxidase (mM)	Additions	Succinic dehydrogenase: Inhibition (%)	Succinic dehydrogenase: Protection (%)
		0·17 mM mapharside		
13·4	—	None	79·0	—
13·4	—	0·064 M phosphate	62·5	20·8
13·4	—	4·0 mM fluoride	63·1	20·1
13·4	—	0·064 M phosphate + 4·0 mM fluoride	10·3	86·6
		0·23 mM melarsen oxide		
26·0	0·5	None	80·0	—
26·0	0·5	0·064 M phosphate	75·2	6·0
26·0	0·5	4·0 mM fluoride	62·0	22·5
26·0	0·5	0·064 M phosphate + 4·0 mM fluoride	0·0	100·0
		8·2 mM iodoacetate		
6·9	0·5	None	76·1	—
6·9	0·5	0·080 M phosphate	70·5	7·3
6·9	0·5	4·0 mM fluoride	70·5	7·3
6·9	0·5	0·080 M phosphate + 4·0 mM fluoride	−6·9	108·7
		0·21 mM *o*-iodosobenzoate		
15·1	0·3	None	75·0	—
15·1	0·3	0·064 M phosphate	43·5	42·0
15·1	0·3	4·0 mM fluoride	53·8	28·2
15·1	0·3	0·064 M phosphate + 4·0 mM fluoride	11·1	85·0
		0·42 mM *o*-iodosobenzoate		
15·1	0·3	None	93·0	—
15·1	0·3	0·064 M phosphate	92·0	1·1
15·1	0·3	4·0 mM fluoride	94·5	−1·6
15·1	0·3	0·064 M phosphate + 4·0 mM fluoride	8·5	90·7
		0·36 mM *N*-ethylmaleimide		
12·0	0·1	None	100·0	—
12·0	0·1	0·060 M phosphate	86·5	13·5
12·0	0·1	4·0 mM fluoride	96·0	4·0
12·0	0·1	0·060 M phosphate + 4·0 mM fluoride	25·0	75·0

Table 2. *Effect of several concentrations of fluoride and phosphate on succinic-dehydrogenase inactivation by N-ethylmaleimide*

1·0 ml. of enzyme suspension (15·2 mg. of succinoxidase) was treated at 37° for 30 min. with 0·48 mM *N*-ethylmaleimide, 0·08 M phosphate, pH 7·3 (Expt. *A*), or 10·0 mM fluoride (Expt. *B*) and additions as shown, and then diluted with 4·0 ml. of distilled water. Succinic dehydrogenase was measured in 0·3 ml. of this extract.

Expt.	Additions	Succinic-dehydrogenase: Inhibition (%)	Succinic-dehydrogenase: Protection (%)
	Fluoride concn. (mM)		
A	None	83·5	—
	10·0	7·0	91·5
	5·0	29·0	65·0
	1·0	68·5	18·0
	0·5	72·0	13·8
	Phosphate concn. (mM)		
B	80·0	18·2	81·8
	20·0	32·4	67·6
	4·0	56·0	44·0
	0·2	100·0	—

Requirement of phosphate for fluoride action. The concentration of phosphate needed to allow the protection of succinic dehydrogenase by fluoride appears to mean a significant increase of ionic strength and molar concentration in the medium where the enzyme reacts with the thiol detectors. This raises the question whether phosphate potentiates fluoride in a non-specific manner, i.e. through changes in the activity coefficient of fluoride ions and the ion groups of succinic dehydrogenase able to bind fluoride. However, the following facts conflict with that hypothesis: (1) sulphate and arsenate potentiate fluoride, but far less than the same concentration of phosphate (Tables 1–4); (2) the change in molarity (and ionic strength) brought about by the addition of phosphate is less striking than appears at first. In the experiments shown in Tables 1 and 2, 0·8 ml. of succinoxidase in 0·2 M borate buffer was treated with 0·2 ml. of ion mixture and 0·02 ml. of inhibitor solution, which means that the maximal increase in molarity was from 0·20 to 0·28; (3) potassium nitrate or sodium chloride, in amount giving the same ionic strength

as phosphate, scarcely affects the action of fluoride (Table 5). There is a certain protection of succinic dehydrogenase by nitrate, which, however, does not potentiate fluoride, as the effect of nitrate and fluoride together exceeds only slightly the sum of their individual actions.

The ionic strength of the ion mixtures added to the succinoxidase–borate suspension has been calculated as half the sum of the products of each ion molarity by the square of its valency (Lewis & Randall, 1923); for the present purpose this may be considered accurate enough. The action of fluoride in media of equal ionic strength is obtained by comparing the experiments with 0·163 M nitrate and fluoride (Table 5), and the experiments with 0·064 M phosphate, arsenicals and *o*-iodosobenzoate, where the ionic strength added to the succinoxidase–borate solution was about 0·16. The action of fluoride in media of equal molarity may be seen by comparing the experiments with 0·060 M chloride (Table 5) with the experiments with 0·060 M phosphate and *N*-ethylmaleimide as inhibitor.

The possibility of replacing phosphate by sulphate, though limited, suggests that phosphate and

Table 3. *Effect of halogen ions on succinic-dehydrogenase inactivation by o-iodosobenzoate*

Enzyme suspension (1·0 ml.) (15·3 mg. of succinoxidase) was treated with *o*-iodosobenzoate, phosphate and additions as shown, for 20 min. at 37°, and was then diluted with 4·0 ml. of distilled water. Succinic-dehydrogenase activity was measured in 0·3 ml. of this extract.

Additions	Succinic-dehydrogenase Inhibition (%)	Protection (%)
0·28 mM *o*-iodosobenzoate + 0·08 M phosphate		
None	62·0	—
4·0 mM fluoride	9·0	85·5
4·0 mM chloride	79·0	−27·4
4·0 mM bromide	78·6	−26·8
4·0 mM iodide	56·5	8·8
4·0 mM thiocyanate	60·0	3·2
0·28 M *o*-iodosobenzoate + 0·2 mM phosphate		
None	100·0	—
4·0 mM fluoride	88·2	11·8
4·0 mM chloride	95·5	4·5
4·0 mM bromide	89·0	11·0
4·0 mM iodide	84·5	15·5
4·0 mM thiocyanate	88·0	12·0

Table 4. *Protection of succinic dehydrogenase by fluoride in the presence of sulphate and arsenate*

Succinoxidase (1 ml.) was treated at 37° with inhibitors and additions as shown for 30 min. (Expt. *A*) or 20 min. (Expt. *B*) and then diluted with 4·0 ml. of 0·4 M phosphate, pH 7·3 (Expt. *A*) or 4·0 ml. of distilled water (Expt. *B*). Succinic dehydrogenase was measured in 0·3 ml. of this extract.

Expt.	Succinoxidase (mg.)	Residual phosphate in succinoxidase (mM)	Additions	Succinic-dehydrogenase Inhibition (%)	Protection (%)
			0·17 mM mapharside		
A	13·4	—	None	79·0	—
	13·4	—	0·040 M sulphate	78·5	0·6
	13·4	—	4·0 mM fluoride	63·1	20·1
	13·4	—	0·040 M sulphate + 4·0 mM fluoride	36·0	54·5
			0·42 mM *o*-iodosobenzoate		
	15·1	0·3	None	93·0	—
	15·1	0·3	0·064 M sulphate	93·5	0·5
	15·1	0·3	4·0 mM fluoride	94·5	−1·6
	15·1	0·3	0·064 M sulphate + 4·0 mM fluoride	49·0	47·2
			0·36 mM *N*-ethylmaleimide		
	12·0	0·1	None	100·0	—
	12·0	0·1	0·060 M sulphate	90·6	9·4
	12·0	0·1	4·0 mM fluoride	96·0	4·0
	12·0	0·1	0·060 M sulphate + 4·0 mM fluoride	40·0	60·0
			0·36 mM *N*-ethylmaleimide		
B	12·0	1·2	None	97·0	—
	12·0	1·2	0·080 M arsenate	98·0	−1·0
	12·0	1·2	4·0 mM fluoride	89·0	8·2
	12·0	1·2	0·080 M arsenate + 4·0 mM fluoride	48·5	50·0
			12·0 mM iodoacetate		
	12·0	1·2	None	90·5	—
	12·0	1·2	0·080 M arsenate	94·0	−3·9
	12·0	1·2	4·0 mM fluoride	89·0	1·7
	12·0	1·2	0·080 M arsenate + 4·0 mM fluoride	46·5	48·5

arsenate potentiate fluoride through their bivalent anions.

Influence of sulphate, nitrate and chloride on inhibition of succinic dehydrogenase by fluoride. It is known that phosphate and arsenate are required if fluoride is to inhibit succinic dehydrogenase (Slater & Bonner, 1952). The action of phosphate has been now compared with that of sulphate, nitrate and chloride (Table 6). As was expected, sulphate increases fluoride action but to a less extent than does phosphate. Nitrate and chloride have no action in amounts giving the same ionic strength as phosphate, e.g. the experiment with 0·10 M phosphate and 0·26 M chloride (added ionic strength 0·26) and the experiment with 0·066 M phosphate and 0·158 M nitrate (added ionic strength about 0·16). Incidentally, the strong inhibition of succinic dehydrogenase by the univalent anions may be noted.

Table 5. *Action of fluoride on succinic dehydrogenase in the presence of nitrate and chloride*

Succinoxidase (1 ml.) was treated with inhibitors and additions as shown for 30 min. at 37° and then diluted with 5·0 ml. (for iodoacetate) or 4·0 ml. (for other reagents) of distilled water. Succinic dehydrogenase was measured in 0·3 ml. of this extract.

Succinoxidase (mg.)	Residual phosphate in succinoxidase (mM)	Additions	Succinic-dehydrogenase Inhibition (%)	Succinic-dehydrogenase Protection (%)
		0·28 mM *o*-iodosobenzoate		
15·3	0·21	None	95·0	—
15·3	0·21	0·163 M nitrate	81·5	14·2
15·3	0·21	4·0 mM fluoride	86·6	8·8
15·3	0·21	0·163 M nitrate + 4·0 mM fluoride	67·5	29·0
		8·0 mM iodoacetate		
18·4	0·27	None	100·0	—
18·4	0·27	0·120 M chloride	96·5	3·5
18·4	0·27	4·0 mM fluoride	96·0	4·0
18·4	0·27	0·120 M chloride + 4·0 mM fluoride	96·5	3·5
		0·28 mM *o*-iodosobenzoate		
11·3	0·14	None	94·0	—
11·3	0·14	0·060 M chloride	97·0	−3·2
11·3	0·14	4·0 mM fluoride	95·0	−1·1
11·3	0·14	0·060 M chloride + 4·0 mM fluoride	94·0	0·0
		0·24 mM *N*-ethylmaleimide		
11·3	0·14	None	100·0	—
11·3	0·14	0·060 M chloride	95·5	4·5
11·3	0·14	4·0 mM fluoride	100·0	0·0
11·3	0·14	0·060 M chloride + 4·0 mM fluoride	86·5	13·5
		0·28 mM *o*-iodosobenzoate		
15·3	0·21	None	97·5	—
15·3	0·21	0·163 M chloride	87·5	10·3
15·3	0·21	4·0 mM fluoride	88·2	9·5
15·3	0·21	0·163 M chloride + 4·0 mM fluoride	85·5	12·3

Table 6. *Effect of phosphate, sulphate, nitrate and chloride on succinic-dehydrogenase inhibition by fluoride*

0·021 M succinate; 0·005 M methylene blue; 0·006 M cyanide; 0·07 M borate buffer, pH 7·3. Air as the gas phase. 37°.

Succinoxidase (mg.)	Additions	Succinic-dehydrogenase inhibition (%)
1·7	10·0 mM fluoride	7·6
	0·1 M phosphate	13·8
	10·0 mM fluoride + 0·1 M phosphate	43·5
	0·1 M sulphate	20·0
	10·0 mM fluoride + 0·1 M sulphate	45·5
	0·26 M chloride	56·2
	10·0 mM fluoride + 0·26 M chloride	58·6
1·9	10·0 mM fluoride	13·7
	0·066 M phosphate	0·0
	10·0 mM fluoride + 0·066 M phosphate	41·0
	0·158 M nitrate	28·5
	10·0 mM fluoride + 0·158 M nitrate	31·0

This has already been observed by Batelli & Stern (1911) with chloride, and by Slater & Bonner (1952) with nitrate.

Inactivation of succinoxidase with BAL in the presence of fluoride. The oxidation of succinoxidase with BAL inactivates a factor linking the dehydrogenase to the cytochrome system (Slater, 1949*b*). It is still under discussion whether the BAL-sensitive factor (Slater's factor) is a distinct enzymic constituent of succinoxidase (Clark, Neufeld, Widmer & Stotz, 1954) or rather a functional group of succinic dehydrogenase (cf. Green & Beinert, 1955), which in the presence of adequate hydrogen acceptors could oxidize succinate notwithstanding the inactivation of the BAL-sensitive factor. According to Slater (1949*c*), succinate and competitive inhibitors of succinic dehydrogenase prevent the effect of BAL on succinoxidase. This remote action of succinate and analogues is of considerable interest, as the 'protection phenomena' would no longer prove that there are thiols in the active centre of succinic dehydrogenase, as suggested by Hopkins *et al.* (1938) and others. On this ground the action of fluoride and phosphate on the inactivation of succinoxidase with BAL seemed worth trying. As shown in Table 7 the association of fluoride and phosphate does not modify the effect of BAL. Experiment *A* is particularly significant, as the partial inactivation of the enzyme rules out the possibility that too long oxidation with BAL had nullified a difference due to the presence of fluoride. The slight protection of succinoxidase shown in that experiment is negligible if it is remembered that the same mixture of phosphate and fluoride prevents completely succinic-dehydrogenase inactivation by *o*-iodosobenzoate and other thiol reagents.

Action of fluoride on succinic dehydrogenase in the presence of ethylenediaminetetraacetate ions. Besides its action on succinic dehydrogenase, quoted above, phosphate strongly activates the oxidation of succinate by molecular oxygen, as it apparently promotes a closer association of succinic dehydrogenase to the cytochrome *c*–cytochrome oxidase system (Bonner, 1954). Ethylenediaminetetraacetate has a similar activating action on succinoxidase, though in much smaller concentrations (Bonner, 1954). The functional analogy between ethylenediaminetetraacetate and phosphate does not extend, however, to the promotion by the latter of succinic-dehydrogenase protection by fluoride, as shown in Table 8. If this related to the lack of action of fluoride on succinoxidase oxidation with BAL and the prevention of the effect of BAL by ethylenediaminetetraacetate (Stopanni & Brignone, in preparation), it may be concluded that agents able to control the activity of succinic dehydrogenase, on the one hand, and the mutual accessibility of succinoxidase components, on the other, have very

Table 7. *Inactivation of succinoxidase with BAL in the presence of fluoride and phosphate*

Succinoxidase (1 ml.) was oxidized with 8·0 mM BAL and additions as shown in Warburg flasks at 37° for 27 min. (Expt. *A*) or 25 min. (Expt. *B*) and then diluted with 10 ml. of distilled water (Expt. *A*) or 5·0 ml. 0·08 M phosphate buffer, pH 7·3 (Expt. *B*). Succinoxidase activity was measured in 0·4 ml. (Expt. *A*) or 0·3 ml. (Expt. *B*) of extract.

Expt.	Succinoxidase (mg.)	Additions	Oxygen taken up in BAL oxidation (μl.)	Succinoxidase inhibition (%)
A	36·0	0·080 M phosphate	72	39·8
	36·0	0·080 M phosphate + 4·0 mM fluoride	91	36·2
B	28·4	0·080 M phosphate	101	91·5
	28·4	0·080 M phosphate + 4·0 mM fluoride	108	96·1

Table 8. *Effect of fluoride on succinic-dehydrogenase inactivation in the presence of ethylenediaminetetraacetate*

Succinoxidase (24·4 mg. in 1·0 ml.), was treated with 0·43 mM *o*-iodosobenzoate and additions as shown for 30 min. at 37° and then diluted with 4·0 ml. of distilled water. Succinic-dehydrogenase activity was measured in 0·3 ml. of this extract. Residual phosphate concentration in the initial enzyme suspension, 0·2 mM.

Additions	Succinic-dehydrogenase	
	Inhibition (%)	Protection (%)
None	91·8	—
1·95 mM ethylenediaminetetraacetate	88·5	3·6
4·0 mM fluoride	92·5	−0·7
1·95 mM ethylenediaminetetraacetate + 4·0 mM fluoride	83·5	9·0

different sites of action. Phosphate may be singled out as an exception, as it can affect the succinoxidase components in several ways which are not necessarily interdependent.

DISCUSSION

The protection of thiol groups of succinic dehydrogenase by fluoride in the presence of phosphate, in proportion to the concentration of both phosphate and fluoride, is a good confirmation of the mechanism proposed by Slater & Bonner (1952) to explain inhibition of succinic dehydrogenase by fluoride.

Succinate and competitive inhibitors of succinic dehydrogenase, on the one hand, and sulphydryl reagents, on the other, are mutually exclusive with regard to succinic dehydrogenase. Since the work of Hopkins *et al.* (1938), this has been taken as evidence for location of the enzyme thiol on the active area of the enzyme, and deductions made from molecular models have led Potter & DuBois (1943) to postulate that the thiol is placed between two –NH– groups with affinity for the carboxylate ions of succinate and some analogues. After binding the enzyme, thiol reagents hinder the access of the substrate to the affinity points and produce inhibition, and the enzyme-bound substrate prevents sulphydryl reagents from reaching the thiols. Slater's (1949*c*) claim that 'protection phenomena' have no topical value for the area of succinoxidase shielded by succinate and competitive inhibitors is not substantiated by our observations with fluoride and similar ones with suramin (Stoppani & Brignone, in preparation). The possibility that a comparatively smaller affinity of these protectors for succinic dehydrogenase prevents their showing their action on the BAL-sensitive group can be ruled out, as the dissociation constants of the fluoride–(enzyme–phosphate), phosphate–(enzyme–fluoride) and suramin–enzyme complexes are respectively $6{\cdot}7 \times 10^{-5}$ and $3{\cdot}3 \times 10^{-4}$ (Slater & Bonner, 1952) and approx. 10^{-6} (Stoppani & Brignone, in preparation), i.e. about or below the values for the dissociation constants of the complexes of the enzyme with succinate and malonate (cf. Slater & Bonner, 1952; Thorn, 1953) which are supposed to prevent the enzyme oxidation with BAL (Slater, 1949*c*).

The remarkable specificity of fluoride and the requirement of bivalent ions, in particular phosphate, is difficult to explain without a better knowledge of the structure of succinic dehydrogenase. The same arguments considered by Slater & Bonner (1952) in relation to the enzyme inhibition by these ions still apply. The presence of haematin and non-haematin iron in some purified preparations of succinic dehydrogenase reported recently by Green, Kohout & Mii (1954) and Singer & Kearney (1954) suggests the possibility of the formation of complexes that could explain the binding of fluoride and phosphate by this enzyme.

SUMMARY

1. Fluoride and phosphate together decrease markedly the inactivation of succinic dehydrogenase by thiol reagents, such as arsenicals, *o*-iodosobenzoate, iodoacetate and *N*-ethylmaleimide.

2. Chloride, bromide, iodide and thiocyanate do not replace fluoride. Sulphate and arsenate can replace phosphate to some extent, but nitrate, chloride and ethylenediaminetetraacetate do not.

3. Fluoride and phosphate do not prevent inactivation of succinoxidase by 2:3-dimercaptopropanol (BAL).

4. Sulphate increases the inhibition of succinic dehydrogenase by fluoride, but has less effect than phosphate. Nitrate and chloride do not promote the inhibition.

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The Use of Homocysteine in the Estimation of Dehydroascorbic Acid

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Two main methods are in current use for the estimation of dehydroascorbic acid. In the one, developed by Roe and his colleagues (Roe & Kuether, 1943; Roe, Mills, Oesterling & Damron, 1948), the dehydroascorbic acid is condensed with 2:4-dinitrophenylhydrazine and the product treated with sulphuric acid to give a red colour, the intensity of which is measured photoelectrically. In a second technique (Tillmans, Hirsch & Siebert, 1932; Eekelen, Emmerie, Josephy & Wolff, 1933; Bessey, 1938), dehydroascorbic acid is measured as ascorbic acid after reduction with hydrogen sulphide and removal of excess of reductant. Disadvantages are associated with both methods; they are non-specific for dehydroascorbic acid, time- and labour-consuming, and in the method involving reduction with hydrogen sulphide it may be difficult to ensure the removal of excess of reductant before estimating the ascorbic acid formed with 2:6-dichlorophenol-indophenol.

In this paper an account is given of a new method, based on the two findings that homocysteine rapidly reduces dehydroascorbic acid at pH 7·0, and that under certain simple conditions 2:6-dichlorophenol-indophenol can be used to estimate ascorbic acid in the presence of homocysteine without interference from the latter compound.

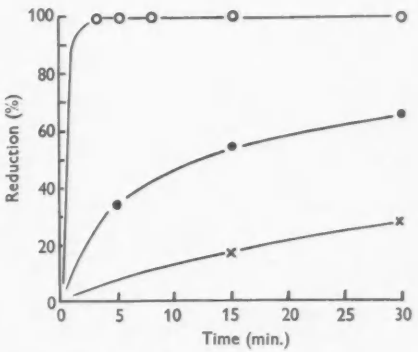

Fig. 1. Reduction of dehydroascorbic acid (0·045 mg./ml.) by various sulphydryl compounds in phosphate buffer of pH 7·2, a molecular ratio (reductant to dehydroascorbic acid) of 40:1 being present in each case. ○, Homocysteine; ●, glutathione; ×, cysteine.

EXPERIMENTAL

Preliminary observations

Reduction of dehydroascorbic acid by homocysteine. At pH values greater than 6·8 homocysteine rapidly reduces dehydroascorbic acid to ascorbic acid. In Fig. 1 the reduction at pH 7·2 of dehydroascorbic acid by three sulphydryl compounds (homocysteine, glutathione and cysteine) is recorded: reduction by homocysteine is complete after a few minutes at room temperature (about 18°), whereas glutathione and cysteine act much more slowly. It was found difficult to measure accurately the rate of the reaction in the presence of cysteine owing to the rapid rate at which cysteine reduces the indophenol dye used in the estimation of the ascorbic acid formed. In these, as in all the experiments described in this paper, the ascorbic acid was measured by the photoelectric method of Bessey (1938).

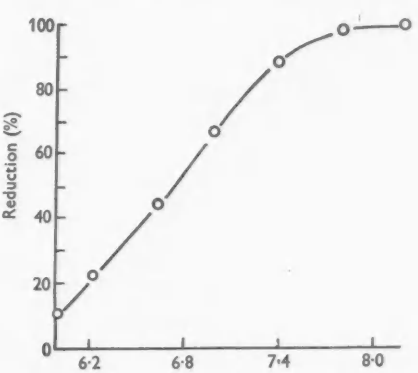

Fig. 2. Percentage reduction, at different pH values, of 0·05 mg. of dehydroascorbic acid by 2·0 mg. of homocysteine. The total volume of the reaction mixture was 6 ml. and the time 8 min.

Table 1. *Effect of different homocysteine:dehydroascorbic acid ratios on the degree of reduction of dehydroascorbic acid*

Time (min.)	Percentage reduction of dehydroascorbic acid: Expt. 1. Molecular ratio homocysteine:dehydroascorbic acid = 10	Expt. 2. Molecular ratio homocysteine:dehydroascorbic acid = 28
3	68	89
6	82	96
10	91	98
15	94	98

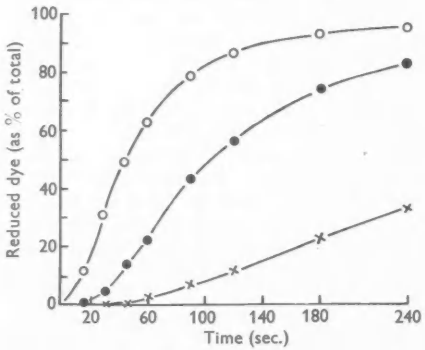

Fig. 3. Reduction of 4 ml. of 2:6-dichlorophenolindophenol dye (1 ml. ≡ 0·02 mg. ascorbic acid) by 0·40 mg. of homocysteine at different pH values. ○, Na_2HPO_4–citric acid buffer, pH 4·5; ●, Na_2HPO_4–citric acid buffer, pH 3·5; ×, Na_2HPO_4–citric acid buffer, pH 2·5.

Below pH 6·8 the rate of the reduction of dehydroascorbic acid by homocysteine is measurably slower; Fig. 2 gives the percentage reduction occurring after 8 min. in buffered solutions of different pH values, the reduction being stopped after 8 min. by acidifying the reaction mixture with 30% (w/v) metaphosphoric acid. For the experiments described above a relatively high homocysteine:dehydroascorbic acid ratio was maintained; this is necessary for complete reduction of the dehydroascorbic acid. Table 1 records the effect of two such ratios upon the reduction of dehydroascorbic acid.

Reduction of 2:6-dichlorophenolindophenol by homocysteine. Sulphydryl compounds reduce the indophenol dye used in the estimation of ascorbic acid at a rate that is dependent upon the pH of the reaction mixture (Mapson & Harris, 1947; Bland, Constable, Harris & Hughes, 1951). It was important to examine the rate of the reduction of the dye by homocysteine in order to ascertain whether the homocysteine would be likely to interfere in the estimation of the ascorbic acid formed. Fig. 3 shows the reaction between 2:6-dichlorophenolindophenol and homocysteine at three pH values, and it will be observed that at pH 2·5 there is negligible reduction of the dye within the first minute; at this pH ascorbic acid reacts with the dye to completion within 2·5 sec. (Mapson & Harris, 1947; Bland *et al.* 1951). This means that homocysteine will not interfere in the estimation of ascorbic acid with 2:6-dichlorophenolindophenol, provided that the reaction is carried out at pH 2·5 and that the galvanometer is read within 30 sec. of the addition of the dye. Estimating the ascorbic acid at a pH below 2·5 would still further reduce the possibility of interference by homocysteine, but would, on the other hand, introduce an error due to the spontaneous fading of the dye which occurs sufficiently rapidly below pH 2·0 to be a source of error.

These findings form the basis of a method for the estimation of dehydroascorbic acid, a preliminary account of which has already appeared (Hughes, 1954*a*).

METHOD

Reagents. (*a*) Homocysteine (Light and Co. Ltd.) (2·0 mg./ml.). (*b*) 2:6-Dichlorophenolindophenol (1 ml. ≡ 0·02 mg. of ascorbic acid). This was usually prepared by diluting a stock solution of which 1 ml. is equivalent to 0·40 mg. of ascorbic acid. The exact strength of the stock solution is determined initially and checked from time to time by titration against a standard, freshly prepared solution of ascorbic acid in 4% metaphosphoric acid. (*c*) Buffer of pH 2·3, 500 ml. of which contains 188·0 g. of citric acid and 30·8 g. of anhydrous Na_2HPO_4. (*d*) 3% (w/v) metaphosphoric acid, used as extractant. (*e*) 45% (w/v) K_2HPO_4 (pure; British Drug Houses, Ltd.). (*f*) Dehydroascorbic acid (used in checking the method). This was prepared by oxidizing ascorbic acid with bromine and removing the excess of bromine by bubbling air through the solution (Association of Vitamin Chemists, 1947).

Procedure. The tissue to be examined is extracted with 3% metaphosphoric acid (or, for plasma, with a solution containing 2% of oxalic acid and 4% of metaphosphoric acid), and a clear solution is obtained by centrifuging and filtration. The method involves the estimation of ascorbic acid in portions of this extract before and after treatment with homocysteine; the difference between the two values, after correcting for volume changes, gives the dehydroascorbic acid content.

Estimation of the ascorbic acid content of the extract. This is estimated by measuring photoelectrically the reduction of a known amount of the indophenol dye by a portion of the solution by a method that is essentially that described by Bessey (1938) being used. Ostwald blow-out pipettes are used to measure 4 ml. of the buffer (pH 2·3) and 2 ml. of the extract into one of the 10 ml., 3 cm. cells of a photoelectric colorimeter. A direct-reading photoelectric colorimeter, with a spot galvanometer, sensitivity of 170 mm./μA, and a 61 N Kodak filter (max. transmission at 520 mμ.), was employed. With water in the second cell, the galvanometer is set to 100, and 4 ml. of the indophenol dye (of which 1 ml. ≡ 0·02 mg. of ascorbic acid) is blown into the cell containing the buffered extract. The mixture is stirred with a small glass rod, the reaction cell moved into position and the galvanometer read within 30 sec. of the addition of the dye. The amount of ascorbic acid is calculated by reference to a standard curve constructed by treating known amounts of ascorbic acid with the dye under the conditions of the estimation. Turbidity, if present, may be corrected for by decolorization as described by Bessey (1938).

Estimation of ascorbic acid + dehydroascorbic acid. The next step is the estimation of the total ascorbic acid present after the conversion of any dehydroascorbic acid present into ascorbic acid with homocysteine. A portion (3 ml.) of the extract is treated in a test tube with 1 ml. of the homocysteine solution. The amount of K_2HPO_4 necessary to change the pH to 6·8 is slowly added from a 5 ml. microburette, the test tube being shaken during the addition. The amount of K_2HPO_4 required may be determined beforehand by titrating 3 ml. of the extractant against the K_2HPO_4 in the presence of bromothymol blue; 3 ml. of a solution containing 3% metaphosphoric acid should require about 1·0 ml. of the K_2HPO_4. The reaction mixture is allowed to stand at room temperature for about 15 min., after which period any dehydroascorbic acid present will have been changed to ascorbic acid (see Fig. 4). The total ascorbic acid is estimated by using 2 ml. of the homocysteine-treated solution in the photoelectric procedure outlined above, care being taken to read the galvanometer within 30 sec. of the addition of the dye, so as to avoid interference by the homocysteine. The volume changes are corrected for and the dehydroascorbic acid content is calculated by subtracting the initial value for the ascorbic acid from the total obtained after treatment with the homocysteine.

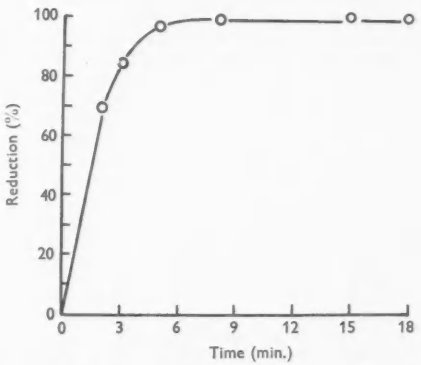

Fig. 4. The rate of reduction of dehydroascorbic acid (0·0048 mg./ml.) by homocysteine. 1 ml. of the homocysteine solution (1·2 mg./ml.) was added to 3 ml. of the dehydroascorbic acid solution, which was then treated with K_2HPO_4 as described in the text. The reaction was stopped after the required time by the addition of 2 ml. of 30% metaphosphoric acid.

If the concentration of ascorbic acid plus dehydroascorbic acid in the extract is low (less than 0·75 mg./100 ml.), then the method may be modified slightly. In such cases 4 ml. of buffer, 3 ml. of the extract and 3 ml. of indophenol dye (strength 1 ml. ≡ 0·01 mg. ascorbic acid) should be used in the estimation, and a homocysteine solution containing 1·3 mg./ml. is sufficiently strong for the reduction of the dehydroascorbic acid.

This technique has been applied to a large number of chemically prepared samples of dehydroascorbic acid and to mixtures of ascorbic acid and dehydroascorbic acid; typical results of such experiments are given in Table 2.

The purity of the potassium phosphate used in the neutralization procedure is of importance; certain samples appear to contain an impurity which, by destroying or removing some of the dehydroascorbic acid, lowers the percentage recovery. Fresh samples of the salt should always be checked by its use in the recovery of standard amounts of chemically prepared dehydroascorbic acid before it is used in the estimation of unknown amounts of the compound.

Specificity of method: the use of boric acid. As is to be expected, the method is not completely specific, certain substances in addition to dehydroascorbic acid being reduced by homocysteine to give reducing products which react with the indophenol dye. Such compounds include alloxan and the products obtained when certain ene-diol substances such as reductic acid, glucoreductone (prepared as described by Kertesz, 1934) and *iso*ascorbic acid

Table 2. *Typical recoveries obtained on applying the homocysteine technique to standard solutions of dehydroascorbic acid and to mixtures of dehydroascorbic acid and ascorbic acid*

Taken		Recovered	
Ascorbic acid (mg./ml.)	Dehydroascorbic acid (mg./ml.)	Ascorbic acid (mg./ml.)	Dehydroascorbic acid (mg./ml.)
0	0·1000	—	0·0960 (96%)
0	0·0800	—	0·0791 (99%)
0	0·0763	—	0·0735 (96%)
0	0·0450	—	0·0441 (98%)
0	0·0382	—	0·0374 (98%)
0	0·0092	—	0·00915 (99%)
0	0·0046	—	0·00452 (98%)
0	0·0047	—	0·00451 (96%)
0·0205	0·0205	0·0202 (99%)	0·0200 (98%)
0·0137	0·0274	0·0137 (100%)	0·0272 (99%)
0·0274	0·0137	0·0278 (101%)	0·0127 (93%)
0·0193	0·0193	0·0187 (97%)	0·0193 (100%)
0·0243	0·0146	0·0247 (101%)	0·0142 (98%)
0·0282	0·0106	0·0290 (102%)	0·0105 (99%)

are oxidized with bromine. These compounds are not believed to be of any biological importance but, even so, some attention was paid to possible improvements by means of which the specificity of the homocysteine reaction could be increased.

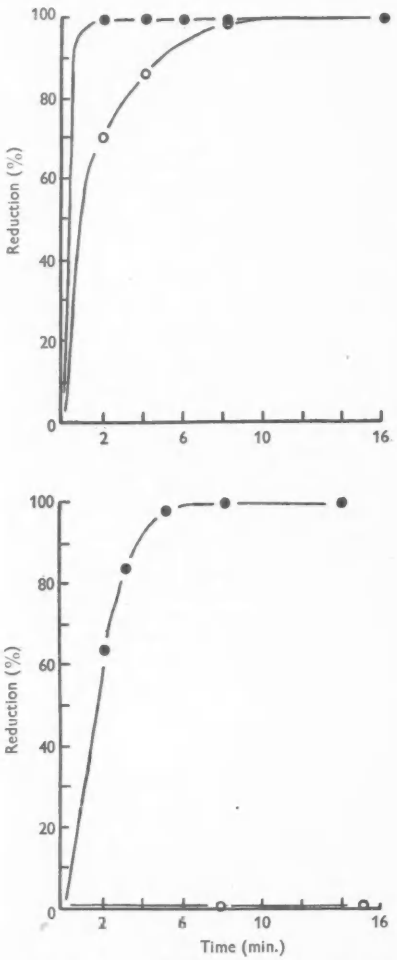

Fig. 5. Effect of boric acid on the reduction by homocysteine of: (*a*) dehydroreductic acid (upper graph) and (*b*) dehydroascorbic acid (lower graph). ●, Normal reduction; ○, reduction in the presence of boric acid. 3 ml. of the dehydroascorbic acid (0·050 mg./ml.) or of the dehydroreductic acid (0·034 mg./ml.) was treated with 1 ml. of homocysteine solution (2·0 mg./ml.) and 1 ml. K_2HPO_4 (30%, w/v) in the presence of 1 ml. aqueous solution of boric acid (about 3%, w/v). The 'normal' values were obtained from a parallel set of experiments in which water was substituted for the boric acid.

It has been known for some time (e.g. Penney & Zilva, 1943; Huelin, 1949) that at alkaline pH values boric acid rapidly destroys dehydroascorbic acid; such a destruction occurs rapidly at pH 7·0, at which the homocysteine reduction is carried out, and if boric acid is added to an acid extract of dehydroascorbic acid there is no formation of ascorbic acid after subjecting such a treated extract to the homocysteine technique. Boric acid, however, has no appreciable destructive effect upon at least some of the interfering compounds listed above. In Fig. 5 the effect of boric acid (in a final concentration of about 0·5%) upon the reduction of dehydroascorbic acid by homocysteine is compared with its effect upon the reduction of dehydroreductic acid (a possible interfering compound) by homocysteine; after 15 min. there is zero reduction of dehydroascorbic acid in the presence of boric acid, whereas the final degree of reduction of the dehydroreductic acid is not appreciably affected, although it may be noted that boric acid causes an initial retardation of the rate. Similar considerations apply with 'dehydroglucoreductone' and alloxan. Hence it can safely be assumed that any compound which is reduced by homocysteine in the presence of boric acid cannot be dehydroascorbic acid. It has been found best when applying this boric acid modification to add 1 ml. of a 3% solution of boric acid to 3 ml. of the acid extract before effecting the change in pH with K_2HPO_4.

As these interfering compounds are believed not to be of widespread occurrence in Nature, it is recommended that in routine estimations of dehydroascorbic acid the unmodified procedure (i.e. without boric acid) be used.

APPLICATIONS

The homocysteine technique has been applied to a number of natural substances, notably to the examination of the dehydroascorbic acid content of plasma and of certain plant tissues. Reference will be made here to the result of one such application, namely the examination of the cotyledonous tissue of germinating peas, a tissue reported by several workers (Robertson, 1947; Sreenivasan & Wandrekar, 1950) to contain comparatively large amounts of dehydroascorbic acid. Such an occurrence of the compound would be unexpected, in view of the findings that pea cotyledons contain a comparatively high concentration of sulphydryl compounds and are also one of the best sources of the enzyme dehydroascorbic acid reductase, which catalyses the reduction of dehydroascorbic acid by glutathione to ascorbic acid (Crook & Morgan, 1944; Hughes, 1954*b*). Accordingly, the dehydroascorbic acid content of pea cotyledons in the initial stages of germination was examined by applying both the homocysteine technique and the dinitrophenylhydrazine method (Roe & Kuether, 1943) to portions of extracts of the cotyledonous tissue, so that a value was obtained for the dehydroascorbic acid by two methods.

Peas were soaked for 12 hr. and allowed to germinate on pads of moist cotton wool covered with gauze. The testas

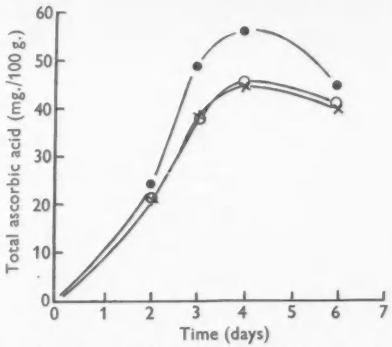

Fig. 6. Formation of dehydroascorbic acid in the cotyledons of germinating peas as measured by the homocysteine and dinitrophenylhydrazine methods. ×, Ascorbic acid by indophenol method; ○, total vitamin C (i.e. dehydroascorbic acid plus ascorbic acid) as measured by the homocysteine method; ●, total vitamin C as measured by the dinitrophenylhydrazine method.

were removed from the peas before germination to enable a greater and more regular formation of ascorbic acid to occur (Hughes, 1954*b*). After timed periods the embryos were removed from 20–30 of the peas and the cotyledons dried between filter paper, weighed and extracted with a solution of 3% metaphosphoric acid. Ascorbic acid was estimated in a portion of the filtered extract and a further portion (3 ml.) was subjected to the homocysteine procedure to obtain a value for the dehydroascorbic acid content. A third portion was diluted with 5% trichloroacetic acid and shaken with charcoal; this was used for the estimation of total vitamin C (i.e. dehydroascorbic acid plus ascorbic acid) by the dinitrophenylhydrazine procedure (Roe & Kuether, 1943).

From the results (Fig. 6) it is seen that there is no increase in ascorbic acid after treatment with homocysteine; this suggests that there is no dehydroascorbic acid present, but there does appear to be present an interfering substance which behaves like dehydroascorbic acid towards the reagents used in the dinitrophenylhydrazine procedure. It is conceivable that the interfering compound is dioxogulonic acid, the first breakdown product of dehydroascorbic acid, but the fact that other workers (Sreenivasan & Wandrekar, 1950) have obtained results which suggest that it is reducible by hydrogen sulphide would appear to militate against this interpretation. The experiment indicates the somewhat greater degree of specificity associated with the homocysteine technique.

DISCUSSION

The time consumption and lack of specificity associated with the methods in current use for the estimation of dehydroascorbic acid have, to some extent, limited the nature and range of investigations into the distribution and metabolism of this substance. The main advantages associated with the technique described in this paper are that there is no need to remove the excess of reductant before measuring the formed ascorbic acid with indophenol dye, and that a single estimation need take no longer than 15 min. at room temperature, as compared with a time of some hours required by other methods. Furthermore, the fact that the reduction occurs rapidly at physiological pH values means that dehydroascorbic acid in certain tissues, plasma and urine can be converted into ascorbic acid by adding homocysteine without any preliminary preparation of an acid extract as would be required in the classical methods; such a direct addition of homocysteine gives information as to the state of any dehydroascorbic acid that might be present, for free dehydroascorbic acid would be changed to ascorbic acid, whereas any 'bound' dehydroascorbic acid would not be reduced until freed by acid extractants. Preliminary work with different plasmas suggests that dehydroascorbic acid exists in plasma in a 'bound' form, possibly in combination with one or more of the proteins of the plasma.

The method, although not in itself completely specific for dehydroascorbic acid, can be made more specific by making use of the knowledge that at pH 7·0 (the pH of the homocysteine reduction) boric acid rapidly and completely destroys dehydroascorbic acid but is without any such effect upon at least some of the interfering compounds.

SUMMARY

1. At pH values above 6·8 homocysteine rapidly reduces dehydroascorbic acid to ascorbic acid.

2. The ascorbic acid so formed can be estimated by reduction of indophenol dye, without any appreciable interference from the excess of homocysteine present, provided that the reaction is carried out within 30 sec. at pH 2·5.

3. Based on these observations, a routine method is described for estimating dehydroascorbic acid which is more rapid and specific than other available methods. The technique involves titration with indophenol dye both before and after the reaction with homocysteine. When the presence of interfering substances is suspected, the specificity can be increased further by titration with and without the addition of boric acid.

4. As an example of the application of the method, tests on pea cotyledons are described.

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